# Novel Biomarkers Associated With Gestational Diabetes Mellitus And Metabolic Outcomes Of Pregnancy

By

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# Dedication

To my husband (Girish) for all his encouragement, understanding and patience during my study

To my children (Nikhil and Ritika) for making it all worthwhile

To my parents and mother-in-law for their assistance and support

## Declaration

I declare that this thesis constitutes my own work and I collected and analysed all the data for the various aspects of the work presented. My collaborators and their contribution to any particular studies have been explicitly stated in the acknowledgement section as well as in the relevant chapters. None of the work has been previously submitted for a higher degree.

# Publications

**Publications arising from thesis**

1. **Sukumar N**, Rafnsson SB, Kandala NB, Bhopal R, Yajnik CS, Saravanan P. Prevalence of vitamin B-12 insufficiency during pregnancy and its effect on offspring birth weight: a systematic review and meta-analysis. **Am J Clin Nutr.** 2016 May;103(5):1232-51
2. **Sukumar N**, Adaikalakoteswari A, Venkataraman H, Maheswaran H, Saravanan P. Vitamin B12 status in women of child-bearing age in the UK and its relationship with national nutrient intake guidelines: Results from two National Diet and Nutrition Surveys. **BMJ Open**. 2016 Aug 12;6(8):e011247.
3. **Sukumar N**, Venkataraman H, Wilson S, Goljan I, Selvamoni S, Patel V, Saravanan P. Vitamin B12 Status among Pregnant Women in the UK and Its Association with Obesity and Gestational Diabetes. **Nutrients** 2016;8(12). doi: 10.3390/nu8120768.

# Synopsis

Gestational diabetes mellitus (GDM), defined as glucose intolerance first identified during pregnancy, is an escalating problem worldwide which affects 5-20% of all pregnant women. It is associated with long-term consequences such as obesity, metabolic syndrome and type 2 diabetes in both the mother and affected offspring, the latter mediated in part by birthweight ("diabetes begets diabetes"). However, selective screening strategies based on established risk factors for GDM, accurately identify only around 60% of cases suggesting that there are other mechanisms involved. The aim of my thesis was to investigate the role of 2 novel biomarkers, vitamin B12 (B12) and glucagon-like peptide (GLP-1) in the development of GDM and related metabolic outcomes.

A systematic review and meta-analysis showed that B12 insufficiency in pregnancy was in the order of 20-30% across the world and was associated with marginally higher, but significant, odds of low birthweight babies but these findings may be isolated to high-risk countries. In a local UK population, B12 insufficiency was independently associated with obesity, 2.6-fold higher risk of GDM and fetal macrosomia. A nationwide survey of women of child-bearing age confirmed that 12% were B12 insufficient with associated hyperhomocysteinaemia, despite apparently adequate dietary intakes of B12. This warrants urgent review of the recommended nutrient intake guidelines to optimise B12 status prior to conception.

In the second part of my thesis, it was shown that overall GLP-1 response during a diagnostic glucose tolerance test is reduced in GDM women compared to controls, with a decrease in the early phase particularly predictive of post-prandial glucose levels. This potentially provides a novel mechanism to explain the delayed first phase insulin response which has been noted in GDM and T2D. Finally, to better understand how GLP-1 may exert a protective effect on the vascular complications of hyperglycaemia, a basic science project was carried out which demonstrated that liraglutide, a GLP-1 receptor agonist, enhanced the AMPK and phospho-AKT signaling pathways thereby contributing to the reduction of oxidative cell damage.

In summary, this thesis supports the hypothesis there are multiple mechanisms which give rise to GDM (e.g. predominant insulin resistance or insulin secretion or combination of factors) and biomarkers such as B12 and GLP-1 can be clinically useful in identification of high-risk women. If proven in larger prospective studies, with measurements of the biomarkers from early pregnancy, these markers may be used to risk-stratify these women with the ultimate goal of reducing the transgenerational perpetuation of diabetes.

## Abbreviations

| | |
|---|---|
| **adoCbl** | Adenosylcobalamin |
| **AGE** | Advanced glycation end-products (AGE) |
| **AMP** | Adenosine monophosphate |
| **AMPK** | 5'-adenosine monophosphate activated protein kinase |
| **ANOVA** | Analysis of variance |
| **ATP** | Adenosine triphosphate |
| **B12** | Vitamin B12 |
| **BAX** | BAX Bcl-2 associated X protein |
| **BMI** | Body mass index |
| **BW** | Birthweight |
| **CaMKK-β** | Calcium/calmodulin-dependent protein kinase kinase-beta |
| **cAMP** | Cyclic adenosine monophosphate |
| **DAG** | Diacylglycerol |
| **DM** | Diabetes mellitus |
| **DNA** | Deoxyribonucleic acid |
| **DOH** | Department of Health |
| **DPP-4** | Dipeptidyl peptidase-4 |
| **EAR** | Estimated Average Requirement |
| **EDTA** | Ethylene diamine tetra-acetic |
| **ELISA** | Electrochemiluminescent assay |
| **eNOS** | Endothelial nitric oxide synthase |
| **FFA** | Free fatty acids |
| **FMD** | Flow mediated dilatation |
| **FOXO** | Forkhead box class O (FOXO) |
| **GDM** | Gestational diabetes mellitus |
| **GIGD** | Gastrointestinal-induced glucose disposal |
| **GIP** | Glucose-dependent insulotropic polypeptide |
| **GLP-1** | Glucagon-like peptide-1 |
| **GLP-1 RA** | GLP-1 receptor agonist |
| **GTT** | Glucose tolerance test |
| **GWG** | Gestational weight gain |
| **HbA1c** | Haemoglobin A1c |
| **Hcy** | Homocysteine |
| **HDL** | High density lipoprotein |
| **HG** | High glucose |
| **holoHC** | Holo-haptocorrin |
| **holoTC** | Holotranscobalamin |
| **HOMA-IR** | Homeostatic model assessment of insulin resistance |
| **HUVECs** | Human umbilical vein endothelial cells |
| **IADPSG** | International Association of the Diabetes and Pregnancy Study Groups |
| **IF** | Intrinsic factor |
| **IFG** | Impaired fasting glucose |
| **IGT** | Impaired glucose tolerance |

| | |
|---|---|
| **IL-6** | Interleukin 6 (IL-6) |
| **ILK** | Intergrin-like kinase (ILK) |
| **IRS** | Insulin receptor substrate |
| **IUGR** | Intrauterine growth retardation |
| **JNK** | c-JUN N-terminal kinases |
| **LBW** | Low birthweight |
| **LG** | L-glucose |
| **LGA** | Large for gestational age |
| **LKB1** | Liver kinase B1 (LKB1) |
| **LV** | Left ventricle |
| **MCM** | Methylmalonyl-CoA mutase |
| **MDM2** | Mouse double minute 2 homolog (MDM2) |
| **methylCbl** | Methylcobalamin |
| **MMA** | Methylmalonic acid |
| **MTHFR** | Methylenetetrahydrofolate reductase |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate hydrogen |
| **NDNS** | National Diet and Nutrition Survey |
| **NF-κB** | Nuclear factor kappa beta |
| **NGT** | Normal glucose tolerance |
| **NICE** | National Institute for Health and Care Excellence |
| **NO** | Nitric oxide |
| **NTD** | Neural tube defects |
| **OR / aOR** | Odds ratio / Adjusted odds ratio |
| **PI3K** | Phosphatidyl-inositol-3 kinase |
| **PIP2** | Phosphatidylinositol-4,5-bisphosphate (PIP2) |
| **PIP3** | Phosphatidylinositol-3,4,5-trisphosphate (PIP3) |
| **PKA** | Protein kinase A |
| **PKC** | Protein kinase C (PKC) |
| **PTEN** | Phosphatase and tensin homolog deleted on chromosome 10 |
| **RCT** | Randomised control trials (RCT) |
| **RDA** | Recommended daily allowance |
| **RIA** | Radioimmunoassay |
| **RNA** | Ribonucleic acid |
| **RNI** | Recommended daily nutrient intake |
| **RNS** | Reactive nitrogen species (RNS) |
| **ROC** | Receiver operating characteristic |
| **ROS** | Reactive oxygen species |
| **SAM** | S-adenosylmethionine |
| **SD** | Standard deviation |
| **SGA** | Small for gestational age |
| **SOD** | Superoxide dismutase |
| **T2D** | Type 2 diabetes mellitus |
| **tAUC** | Total area under the curve |
| **TC** | Transcobalamin |
| **THF** | Tetrahydrofolate |
| **TNF-α** | Tumour necrosis factor α |

| | |
|---|---|
| **VCAM-1** | Vascular-cell adhesion molecule-1 |
| **VEGF** | Vascular endothelial growth factor |
| **WHO** | World Health Organization |

# Chapter 1

# Overview of gestational diabetes mellitus

### 1.1. Metabolic disease in pregnancy

Diabetes mellitus is chronic metabolic disease characterised by hyperglycaemia, which in Type 2 diabetes mellitus (T2D) is due to combination of insulin resistance and beta-cell dysfunction. There is an escalating epidemic of T2D across the world with 380 million people affected in 2013, which is expected to increase to 470 million by 2035 (International Diabetes Federation 2013). The age of diagnosis of T2D is also coming down with more children and young adults being diagnosed with it, which runs in parallel with the increase in childhood obesity (Rocchini 2002). Studies from the Pima Indian group and a Japanese population show a 6-fold and 2-fold increase in the incidence of T2D in children in the last 20-40 years (Fagot-Campagna et al. 2001; Urakami et al. 2005).

Pregnancy is a period of physiological insulin resistance due to placental secretion of diabetogenic hormones such as progesterone, growth hormone and corticotrophin-releasing hormone which ensure an adequate supply of nutrients to the fetus. It is estimated that insulin resistance increases by 50-60% in the latter half of pregnancy (Catalano and Hauguel-De Mouzon 2011) and clinically recognisable diabetes develops when the maternal pancreas is unable to secrete enough insulin to cope with this. Nearly 50% of people in the world with T2D remain undiagnosed (International Diabetes Federation 2013) and therefore pregnancy provides a crucial window of opportunity to identify women at risk and intervention can be provided during pregnancy as well as postnatally.

Gestational diabetes mellitus (GDM) is described as glucose intolerance first recognised in pregnancy. Similar to T2D, GDM is also increasing at an alarming rate, attributed partly to increasing maternal age and body weight (Ferrara et al. 2004; Hedderson et al. 2010). The current estimate is around 4% of all pregnancies (Metzger et al. 2005) though this could increase to as much as 18% if the new International Association of the Diabetes and Pregnancy Study Groups (IADPSG) guidelines are implemented (Moses 2010).

### 1.2. Diagnosis of GDM

There is no internationally accepted method of screening for GDM with disparities in whom to screen, gestation of screening, dose and duration of glucose tolerance test (GTT), and the cut-off levels used. In the UK, the National Institute for Health and Care Excellence (NICE) recommends a 75g 2-hour GTT to be done at 24-28 weeks pregnancy in mother with risk factors (i.e. prepregnancy BMI >30kg/$m^2$, previous macrosomic baby, previous GDM, 1$^{st}$-degree relative with diabetes and ethnic minority groups) (National Institute for Health and Care Excellence 2008). The WHO criteria (fasting plasma glucose ≥ 7.0 and/or 2-hour post-prandial glucose ≥ 7.8mmol/l) is suggested by NICE for the diagnosis of GDM (World Health Organisation 1999), although many Trusts in the UK use the modified WHO criteria with fasting glucose ≥ 6.0mmol/l (Nanda et al. 2011), for which there is no direct evidence. There is a strong case for revising these cut-offs based on data from the large international Hyperglycaemia in Pregnancy and Adverse Outcomes study which showed that the risk of adverse neonatal outcomes (neonatal hypoglycaemia, cord C-peptide >90$^{th}$ centile, C-section rate and birthweight >90$^{th}$ centile) was linear according to maternal plasma glucose level at GTT with no threshold (The HAPO Study Cooperative Research Group 2008). Cut-off values of fasting glucose ≥ 5.1, 1-hour ≥ 10 and 2-hour ≥ 8.5 mmol/l on a 75g GTT were therefore recommended based on a 1.75 relative risk for these outcomes and has been adopted by Scotland and USA (American Diabetes Association 2013; Scottish Intercollegiate Guidelines Network 2010). While this data is compelling, the benefits of treating at these cut-offs is not known due to lack of evidence from intervention studies.

### 1.3. Adverse maternal outcomes of GDM

Mothers with a diagnosis of GDM are more likely to have complications during the pregnancy with higher risk of polyhydramnios (Farooq et al. 2007), hypertension and vaginal candidiasis (Odar et al. 2004), pre-eclampsia (Yogev et al. 2010) and primary caesarean section (The HAPO Study Cooperative Research Group 2008).

Women who develop GDM are at have a relative risk of 7.43 of developing T2D, with the higher risk being seen as early as 5 years after pregnancy (Bellamy et al. 2009). Another systematic review concluded that the 3 most significant factors which

predict the conversion to T2D in these women were suboptimal body anthropometry, poor glycaemic control during pregnancy (use of insulin was associated with a hazards ratio 3.5 for future T2D in Lee et al) (A. J. Lee et al. 2007), and diagnosis of GDM prior to 24 weeks gestation (Baptiste-Roberts et al. 2009).

Additionally, these women are at higher risk of cardiovascular morbidity with the largest study showing significantly higher adjusted odds ratio of around 2.5 for simple cardiovascular events and hospitalisations at 10-20 years post-pregnancy (Kessous et al. 2013).

## 1.4. Offspring outcomes of GDM

### 1.4.1. Perinatal outcomes

Fetal macrosomia (defined as birthweight > 4-4.5kg) and large for gestational age (LGA) (defined as birthweight >2 SD greater than mean or >90$^{th}$ centile after controlling for age and sex) are 2 of the most common and serious outcomes of GDM in pregnancy (Suhonen et al. 2008). Babies born to GDM mothers are 4 - 7 times likely to be macrosomic (Ostlund et al. 2003; Suhonen et al. 2008).

Other perinatal complications which are also associated with hyperglycaemia include shoulder dystocia and birth injuries, neonatal hypoglycaemia and respiratory distress syndrome (The HAPO Study Cooperative Research Group 2008). The complications are at least in part related to glycaemic control because it was found in a meta-analysis that specific treatment of GDM significantly reduced shoulder dystocia, large for gestational age babies and macrosomia by about 60% compared to usual care, although it must be noted that the diagnostic targets for GDM and threshold of treatment varied in the studies (Horvath et al. 2010).

Apart from body weight per se, it is the distribution of fat in fetuses of mothers with GDM that is concerning because it has been shown that these babies are born with greater skinfold thickness and fat mass, which are markers of fetal adiposity, even when the birthweight is appropriate for gestational age, sex and ethnicity (Catalano et al. 2003; Catalano and Hauguel-De Mouzon 2011). Aside from glucose, an

oversupply of nutrients such as triglycerides and free fatty acids may also drive fetal weight gain in these babies (Catalano and Hauguel-De Mouzon 2011).

### 1.4.2. Transgenerational effect

In the long-term, children born to GDM mothers are at increased risk of becoming overweight before puberty, developing central obesity and metabolic syndrome in adolescence and having pre-diabetes or T2D in young adulthood (Damm et al. 2016; Gillman et al. 2003).

Several questionnaire-based studies which have looked into the parental history of diabetic probands found that the individuals were 2 to 4 times more likely to have a mother affected with diabetes than father (Alcolado and Alcolado 1991; Bo et al. 2000). While mitochondrial deoxyribonucleic acid (DNA) mutations in genes that control insulin secretion from the beta-cell are inherited exclusively from a mother, this is likely to only account for a small proportion of the risk of the transmission of T2D (Alcolado et al. 2002).

The focus therefore shifts to the role of the offspring's exposure to a diabetic intrauterine environment, which increases their risk of diabetes and obesity above that which can be attributable to genetic factors (Portha et al. 2011). This was demonstrated in the Pima Indian cohort study where the children who were born to mothers who developed T2D before the pregnancy had a 3.7 higher odds of developing T2D and a mean body mass index (BMI) 2.6kg/$m^2$ higher than their siblings who were born before their mother developed T2D (Dabelea et al. 2000).

It has been shown that daughters born to mothers diagnosed with GDM in their pregnancy had nearly 2-times the odds of obesity and insulin resistance themselves at age 15, even after adjustment for birthweight (Egeland and Meltzer 2010). Therefore it is not surprising that these female individuals are at higher risk of gestational diabetes themselves when they reach childbearing age in their adolescence or early adulthood. This sets up a vicious cycle of metabolic disease in young adults, which can be passed on from one generation to another.

### 1.4.3. Outcomes in children born at extremities of birthweight

It is well known that babies born to mothers with GDM are at high risk of macrosomia and obesity later on in life. However, a notion that babies born at low birthweight (LBW) are at a similarly at high risk of T2D as larger babies is one that has gained acceptance over the last 20 years, in a pattern known as the U-shaped curve (Figure 1.1) (Harder et al. 2007). The thinking behind this is known as the 'thrifty phenotype hypothesis' and in short, proposes that poor nutrition in fetal and early postnatal life impairs the growth of the beta-cells of the pancreas which predisposes an individual to the development of T2D later on (Hales and Barker 1992).

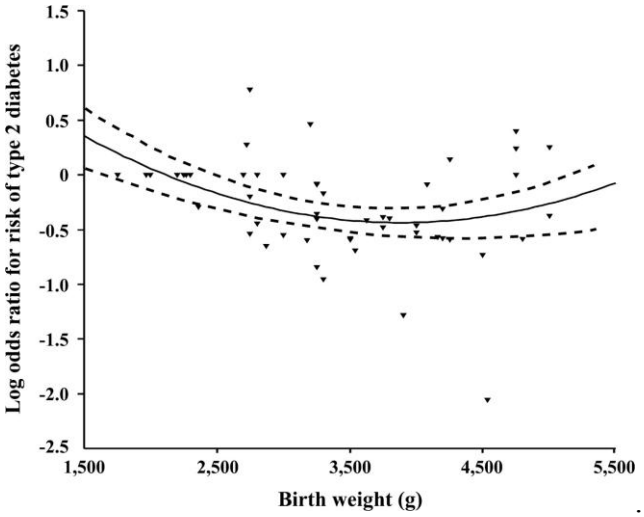


**Figure 1.1** Meta-regression curve of log odds ratios for type 2 diabetes mellitus by birth weight in a meta-analysis (1966–2005). (From Harder et al. 2007).

Several studies from around the world lend support to this theory and the outcome of T2D and metabolic syndrome has been demonstrated from as early as age 4 (C.S. Yajnik et al. 1995) and pre-puberty (Hofman et al. 1997) in individuals born with a low birthweight or ponderal index (weight/length$^3$). A meta-analysis confirmed that the odds ratio (OR) for T2D was 0.76 (95% CI 0.70-0.82) for every 1kg increase in birthweight, after correcting for current BMI (Whincup et al. 2008).

The hypothesis is that the cross-talk between key organs involved in glucose metabolism, namely beta-cells of the pancreas, liver, skeletal muscle and adipocytes, may be affected due to nutritional insults in pregnancy (Maloney and Rees 2005).

Therefore any alteration to the environment in childhood / adulthood, particularly an obesogenic one, can make the individual to the unable to adapt, leading to the development of a disease state (Hales and Barker 2001). Therefore birthweight is likely to be a surrogate for an extremity of nutritional insult in the intrauterine environment which adversely programmes a fetus for postnatal ill-health.

### 1.5. Established maternal risk factors for GDM

There is a close association between maternal obesity and GDM. A meta-analysis of 20 studies found that overweight (BMI 25-29.9 kg/$m^2$), obese (BMI ≥30 kg/$m^2$) and morbidly obese (BMI generally ≥40 kg/$m^2$ but variable in some studies) women had an 2.1-, 3.6- and 8.6-fold increased risk of GDM compared to lean women (Chu et al. 2007). It is not just the maternal BMI which is a predictor of GDM – Martin et al found in small study that higher visceral adipose tissue depth measured by ultrasonography at 12 weeks gestation, was associated with an OR of 17 for GDM diagnosis later in the pregnancy (Martin et al. 2009). No association was seen for subcutaneous fat measurement suggesting that it is the distribution of the fat rather than overall fat mass which is more pathogenic (Martin et al. 2009).

GDM is also related to underlying components of the metabolic syndrome as demonstrated by Retnakaran et al who found that these mothers as well as those with mild glucose intolerance in pregnancy had odds of over 2 of developing metabolic syndrome (as defined by International Diabetes Federation) at 3 months-postpartum (Retnakaran et al. 2010a). However, when prepregnancy BMI and weight gain in pregnancy were added to the model the risk in the latter group lost significance, strengthening the case for the role of obesity.

Being of South Asian origin increases the risk of GDM, even at lower BMI levels (Hedderson et al. 2012) but the reasons for this is unclear. Both a higher fasting glucose (Jenum et al. 2012) and higher postprandial glucose levels (Gunton et al. 2001) are found in this group in different studies. Aside from genetics, other factors which could mediate this risk include lower socio-economic status (maternal height, which is a surrogate marker for deprivation in-utero or early childhood, is a negative

predictor of GDM (Ogonowski and Miazgowski 2010)), distribution of fat (Lear et al. 2007) or as-yet unidentified factors.

If the well-established risk factors were able to accurately predict the risk of GDM, selective screening criteria based on these risk factors should be able to pick-up most of the women GDM. However, selective screening strategies consistently perform poorer than universal screening, with a diagnostic rate of around 60%, and a very high false negative rate of 30-40% (D. A. Scott et al. 2002) suggesting that there are additional factors in the background population mediating the risk.

### 1.6. Novel biomarkers known to be associated with glucose intolerance or metabolic disease in pregnancy

Increased free fatty acids (FFA) and triglycerides are important surrogates of insulin resistance and it is possible that these factors are also involved in the adverse outcomes both for mothers and their offspring, especially in obese individuals (Catalano and Hauguel-De Mouzon 2011). This was confirmed by Brisson et al who showed that a combination of high triglycerides and waist circumference at 1$^{st}$ trimester had an aOR of 4.7 for future diabetes mellitus (DM) (Brisson et al. 2010). Furthermore the risk of high maternal FFA and lipids are also associated with increased neonatal weight (Kitajima et al. 2001) and neonatal adiposity (Schaefer-Graf et al. 2008) in GDM women, independent of glycaemia.

The notion that we need to move away from a 'glucocentric' approach when discussing GDM and its outcomes is also supported by studies on offspring of mothers with GDM. In a follow-up study of children of mothers with diabetes in pregnancy, those offspring whose mothers had GDM had a 1.5 times higher risk of T2D/pre-diabetes at mean age 23 years compared to both offspring of Type 1 diabetes mothers and mothers who had risk factors for GDM but were GTT negative (no-GDM) (Clausen et al. 2008). This suggests that there are additional factors which predispose a woman to developing GDM that also have adverse consequences for offspring. The same authors showed that the risk of offspring metabolic syndrome (defined as central obesity plus any 2 of high triglycerides, low high density lipoprotein (HDL), hypertension or elevated fasting glucose) were 1.5-fold higher in

children of mothers with GDM compared to type 1 diabetes or no-GDM in pregnancy (Clausen et al. 2009). This implies that additional risk factors either on their own or in combination with intrauterine hyperglycaemia confer the higher adverse consequences. In another study, the risk of overweight and abdominal obesity in 16 year old offspring were highest in those had concomitant antenatal exposure to GDM and maternal pre-pregnancy overweight but not for maternal GDM alone again implying that maternal BMI may be a surrogate for factors contributing to this risk (Pirkola et al. 2010).

Several other groups have also attempted to identify various biomarkers associated with increased incidence of GDM. Markers of inflammation such as highly-sensitive C-reactive protein (CRP) and ferritin have been associated with OR of GDM of 2 -3, which is partly mediated by obesity (X. Chen et al. 2006; Wolf et al. 2003). Fat- and liver-derived biomarkers such as adiponectin and sex-hormone binding globulin at 11-14 weeks gestation increased the detection rate of GDM later to nearly 75% compared to 62% for maternal factors alone, with the false positive rate set at 20% (Georgiou et al. 2008; Nanda et al. 2011). Abnormal carbohydrate metabolism in diabetic pregnancies is associated with increased glycosylation of proteins such as fibronectin (C. L. Lee et al. 2011). In a study looking at several biomarkers at early pregnancy including glycosylated fibronectin, adiponectin, high-sensitivity CRP and placental lactogen, fibronectin had the highest sensitivity (81%) and specificity (90%) for detection of GDM although the authors did not include maternal characteristics in the ROC model (Rasanen et al. 2013). With the advent of metabolomic studies, a whole host of new biomarkers associated with GDM are beginning to emerge and we await with interest the results from these studies and potential clinical utility of these markers (S. Finer et al. 2014).

### 1.7. Combined screening tool

The reality is likely to be a combination of factors accounting for the multifactorial pathogenesis of GDM. This was studied by Savvidou et al, who used a combination of biochemical parameters associated with T2D, namely lipid profile, adiponectin, E-selectin (an adhesion molecule), gamma-glutamyl transferase, CRP, and tissue plasminogen activator (t-PA), at 11-13 weeks gestation as predictors of GDM. They

found that the strongest biochemical predictors of GDM were t-PA and low HDL which, when combined with the basic model of risk factor screening increased the AUC-ROC to 0.86 from 0.82, suggesting a higher pick-up rate whilst reducing false-positives (Savvidou et al. 2010).

Therefore it can be seen that while the incidence GDM continues to increase and there is clear evidence of its long-term consequences for both maternal and offspring health, our understanding of its pathogenesis is still incomplete and warrants further study. In my thesis, I would like to discuss the role of 2 novel biomarkers which are potentially associated with this condition and their clinical implications.

# Chapter 2

# Overall aims

The overall research question of my thesis is to evaluate the contributory role of 2 novel biomarkers in the diagnosis of gestational diabetes (GDM) and related materno-fetal outcomes, namely maternal obesity and extremities of fetal birthweight. Of the various biomarkers associated with metabolic disease in pregnancy, the 2 which I have selected to study in depth are vitamin B12 (B12) and glucagon-like peptide-1 (GLP-1).

The justification of selecting these 2 biomarkers are that while the micronutrient B12 is historically known to be essential for normal embryogenesis, its role in non-communicable diseases, such as metabolic disease and obesity, is gaining more attention with emerging studies on epigenetics and intrauterine programming. Consequently, its association with metabolic disease in pregnancy warrants further evaluation. On the other hand GLP-1, an incretin hormone known to stimulate insulin secretion from the pancreas, has been studied in depth in adults with insulin resistance and type 2 diabetes but is less well characterised in GDM, which is considered to be a 'pre-diabetes' state. Therefore, these 2 biomarkers may differentially affect the pathogenic abnormalities in GDM (insulin resistance and insulin secretion respectively), providing insight into our understanding of this condition, which is still incomplete.

Additionally, if my research and further studies do prove an association between these factors and GDM/metabolic outcomes in pregnancy, there are potential treatment options available in the form of B12 supplements and GLP-1 agonists. Of course, clinical studies will first need to be carried out to test the safety and efficacy of these drugs during pregnancy but it is necessary to prove an association, if not causality, prior to any intervention trials.

Prior to investigating the association between low B12 and GDM, my aim is to assess B12 status in 'normal' pregnancy which will be done in a systematic review and meta-analysis of the prevalence of B12 insufficiency in pregnant women across the world and its potential impact on low birthweight. This is followed by a retrospective observational study to look at the relationship between B12 insufficiency with GDM in a local UK population and its effects on extremities of offspring birthweight. I will then examine the adequacy of dietary B12 intake in women of child-bearing age with

the goal of discussing strategies to optimise B12 status in the pre-conception/early pregnancy period. The objectives of the second part of my thesis are to characterise the GLP-1 response during a glucose tolerance test in women who subsequently develop GDM compared to controls, and finally to describe the methodology for a pilot laboratory project exploring mechanisms by which liraglutide, a GLP-1 agonist, can alleviate vascular complications due to hyperglycaemia.

# Chapter 3

# Role of vitamin B12 in cellular metabolism, embryogenesis and pregnancy

## 3.1. Biochemistry and absorption of vitamin B12 in the human body

### 3.1.1. Vitamin B12 structure and cellular function

Vitamin B12 (B12), also known as cobalamin, is a micronutrient essential for cellular growth, differentiation and development (C. S. Yajnik and Deshmukh 2012). Along with folic acid, it is necessary for the synthesis of DNA, RNA, lipids and protein in the cellular cytoplasm (Saravanan and Yajnik 2010; A David Smith et al. 2008).

B12 is composed of a central cobalt atom surrounded by the 4 nitrogen atoms of a corrin ring. Additionally, it contains a dimethylbenzimidazolyl side group at its lower pole and the sixth position of the cobalt atom on its upper pole can be occupied by a variety of groups such as OH- (hydroxocobalamin), CH3- (methylcobalamin), 5'deoxyadenosylcobalamin or CN- (cyanocobalamin, the term B12 commonly refers to this form). However in the human body, B12 is required for 2 cellular reactions as a necessary cofactor in the form of methylcobalamin (methylCbl, the cofactor of methionine synthase) and adenosylcobalamin (adoCbl, the cofactor of methylmalonyl-CoA mutase) (Krautler 2012). Commonly used synthetic preparations of B12 are cyanocobalamin and hydroxocobalamin. These cellular reactions involving B12 are summarised in Figure 3.1 and discussed in more detail below (Figure 3.1) (W. Herrmann and Obeid 2012).

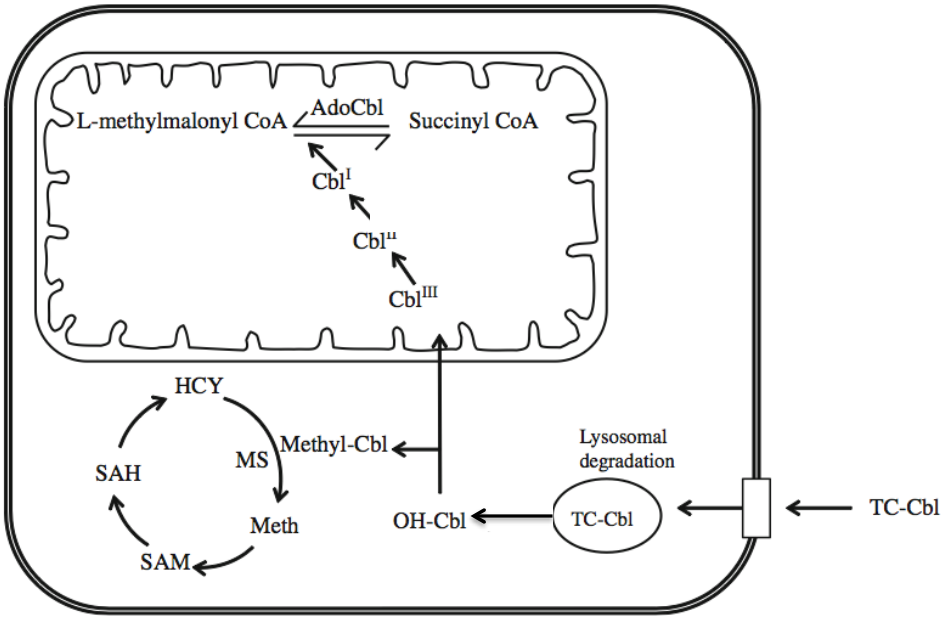


**Figure 3.1** Metabolic pathways enhanced by vitamin B12 or cobalamin (Cbl) (see text for details). TC-Cbl: transcobalamin, Hcy: homocysteine, Meth: methionine, SAH: S-adenosylhomocysteine, SAM: S-adenosylmethionine, methyl-Cbl: methyl cobalamin, Ado-Cbl: adenosyl cobalamin [adapted from Herrmann and Obeid 2012].

Related to B12 is another micronutrient, folate, which is a group of related compounds based on the folic acid structure and is essential for the one-carbon metabolism reactions (see below). It is consumed in the body either as folic acid (used in supplements and fortified foods as it is highly bioavailable and easily reduced to tetrahydrofolate (THF)) or dietary folate (usually in the polyglutamate form).

B12 enters the cytosol as hydroxocobalamin (OHCbl), which becomes methylated to methylCbl after binding to methionine synthase. The first reaction which for which B12 and folate are needed as co-factors is the conversion of homocysteine (Hcy) to methionine by methionine synthase. This is one of many reactions in the one-carbon metabolism cycle, which is so called because a carbon unit is transferred from serine or glycine to THF to form 5,10-methyleneTHF (Johansson et al. 2009; Selhub 2002) (Figure 3.2). 5,10-methyleneTHF can then be used for the formation of thymidine (dTMP, required for DNA), converted to 10-formylTHF (required for synthesis of purines, a component of DNA and RNA) or reduced to methyl-THF by the rate limiting enzyme methylenetetrahydrofolate reductase (MTHFR) (Selhub 2002). Methyl-THF donates its methyl group to Hcy to form methionine by the enzyme methionine synthase, which uses B12 as a co-factor. The methionine thus formed

becomes converted to S-adenosylmethionine (SAM), a universal donor of methyl groups necessary for the formation of DNA, RNA, neurotransmitters, membrane phospholipids, proteins and hormones (Johansson et al. 2009; Saravanan and Yajnik 2010; Selhub 2002).

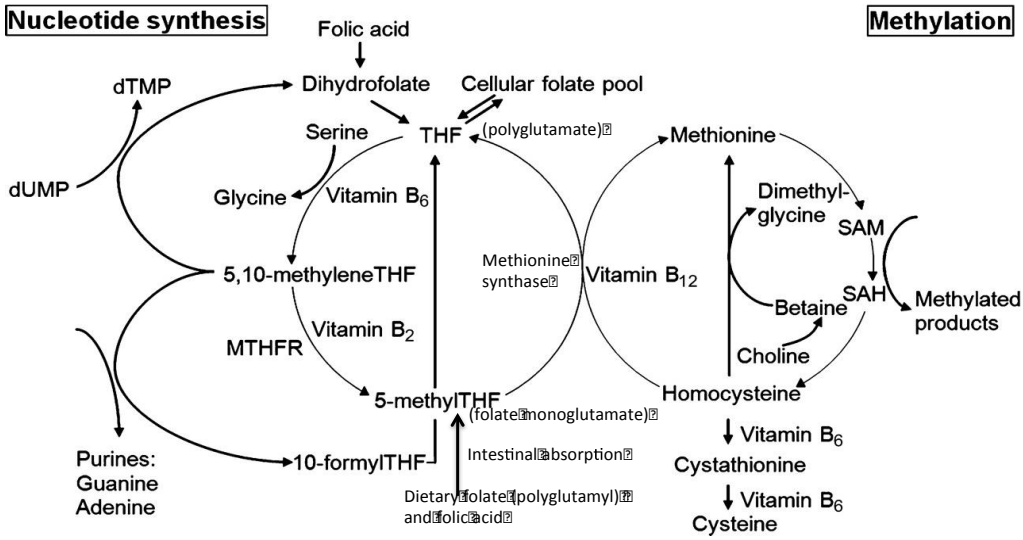


**Figure 3.2** Diagram of the one-carbon metabolism pathway. Labile methyl groups are supplied by dietary serine, choline (via betaine), and methionine. One-carbon units are derived from serine through the activity of the vitamin B6 – dependent serine hydroxymethyltransferase, which generates 5,10-methyleneTHF. 5,10-MethyleneTHF, in turn, is then reduced to 5-methylTHF, the predominant form of folate in the circulation, in an irreversible reaction catalyzed by vitamin B2 – dependent MTHFR. MS serves as a methyl donor in a reaction converting homocysteine to methionine, in which vitamin B12 serves a cofactor. Homocysteine can also be metabolized to cysteine through the sequential action of two vitamin B6 – dependent enzymes [i.e., the transsulfuration pathway]. The methionine derivative, S-adenosylmethionine, is the universal methyl donor for the methylation of a vast variety of molecules, including DNA.
5,10-methyleneTHF: 5,10-methylenetetrahydrofolate, 5-methylTHF: 5-methyltetrahydrofolate, MTHFR: methylenetetrahydrofolate, MS: methinoine synthase [Adapted from Johansson et al. 2009]

In the second reaction which occurs in the mitochondria, methylmalonyl-CoA mutase (MCM), using adoCbl as a co-factor, produces succinyl-CoA from methylmalonyl-CoA. Succinyl-CoA is required for the catabolism of fatty acids, cholesterol and amino acids via the Krebs cycle (W. Herrmann and Obeid 2012). Any excess methylmalonyl-CoA is converted into methylmalonic acid (MMA) so B12 deficiency which would inhibit the conversion of methylmalonyl-CoA to succinyl-CoA, can lead to elevation of MMA that can be detected in plasma.

Broadly speaking, the cellular level effects of B12 deficiency are 2-fold, in keeping with the 2 primary reactions involving B12 as described above (Figure 3.3) (Saravanan and Yajnik 2010). These are:-

1) Deficiency of cytosolic B12: This leads to build up of Hcy in the cell and consequently reduction in methionine and SAM. Lack of SAM leads disrupts the stability of the genome due to hypomethylation of DNA and myelin sheaths (Fenech 2001; Saravanan and Yajnik 2010). Additionally, SAM is an inhibitor of MTFHR, an irreversible rate-limiting enzyme that converts 5,10-methyleneTHF to 5-methylTHF. Reduction in SAM leads to increased MTHFR activity and production of 5-methylTHF, which gets trapped in that form due to B12 deficiency (known as 'folate trap hypothesis') (Das and Herbert 1976; Selhub et al. 2008). The resulting deficiency of nonmethylated forms of folate affects serine to glycine and deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) conversions, causing uracil incorporation into DNA (instead of thymidylate) and increasing risk of chromosomal breakage (Blount et al. 1997) (Figure 3.3).

2) Deficiency of mitochondrial B12: In the absence of B12, build-up of methylmalonyl-CoA and MMA inhibit a key enzyme required for beta-oxidation of fatty acids, known as carnitine palmitoyl transferase 1. The resulting increase in non-oxidised fatty acids can contribute to lipogenesis and insulin resistance especially if impaired glucose metabolism co-exists (Saravanan and Yajnik 2010).

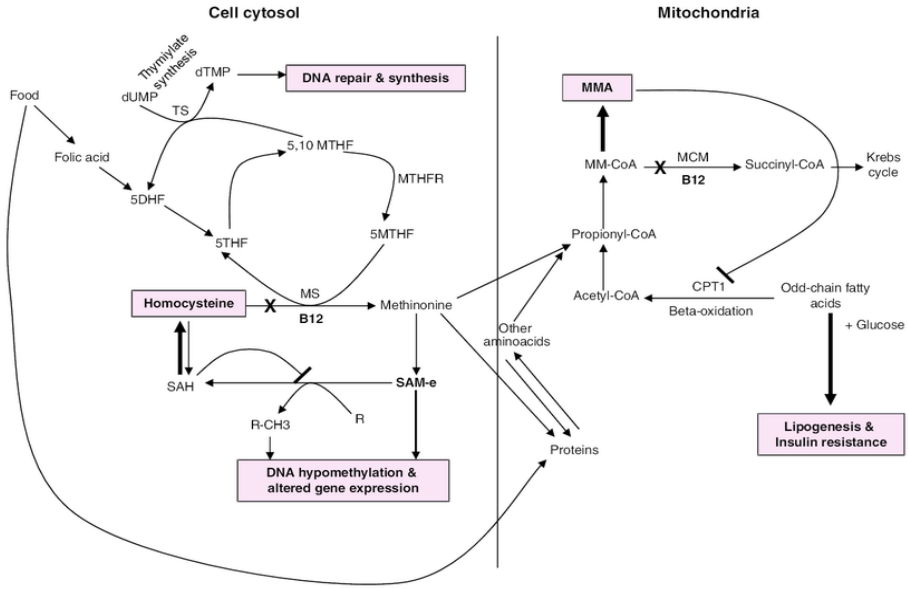


**Figure 3.3** Pathways that involve vitamin B12 and the suggested mechanisms of increased adiposity and insulin resistance in maternal B12 deficiency (see text for details).
dTMP: thymidine monophosphate, dUMP: deoxyuridine monophosphate, TS: thymidylate synthase, MTHF: methyl tetrahydrofolate, MTHFR: methylene tetrahydrofolate reductase, DHF: dihydrofolate, THF: tetrahydrofolate, MS: methionine synthase, SAH: S-adenosylhomocysteine, SAM-e: S-adenosyl methionine, R: methyl acceptor, R–: methylated compound, MM-CoA: methylmalonyl-CoA, MCM : methylmalonyl-CoA mutase, CPT1: carnitine palmitoyltransferase-1 [From Saravanan and Yajnik 2010]

### 3.1.2. Absorption and dietary sources of B12

The absorption of B12 by the human body is complex and involves several processes in the gastro-intestinal tract. These steps as well as causes of B12 deficiency relating to disruption in this absorption process are summarised in Figure 3.4 (Andres et al. 2004). In summary, dietary B12 is released from food in the stomach by pepsin, which functions only in the acidic environment of the stomach. It then binds to R-protein, a B12-transport protein produced in salivary and gastric parietal cells. A key carrier protein for B12 is intrinsic factor (IF) which is also secreted by the gastric parietal cells. Since B12 binding to IF is very weak in the acidic environment of the stomach, B12 enters the duodenum where it is becomes dissociated from the R-protein complex due to the action of pancreatic proteases. Then it binds to IF and this complex is carried down the small intestine until the terminal ileum where it attaches to IF-receptors and is absorbed into the bloodstream by endocytosis. Over 95% of dietary B12 is absorbed through the IF-pathway as described above and only small fraction (1-2%) is passively absorbed via the surface of the intestinal tract.

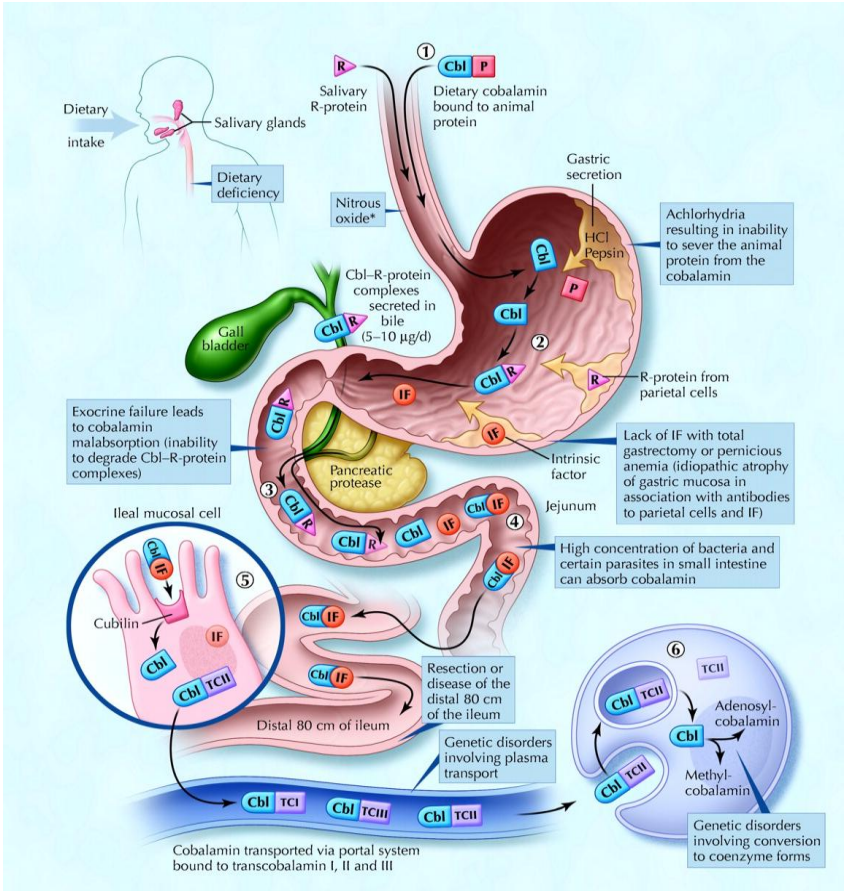

**Figure 3.4** The metabolic pathway of vitamin B12 (cobalamin or Cbl) and corresponding causes of deficiency. (1) Dietary intake of vitamin B12 enters the stomach bound to food protein (P). (2) In the stomach, the acidic environment (pepsin and hydrochloric acid (HCL)) facilitates the cleavage of vitamin B12 that is bound to P, giving the free form of the vitamin. The released form of vitamin B12 is immediately passed to a mixture of glycoprotein such as R-protein (R), which is primarily produced by the parietal and salivary cells, to protect vitamin B12 from chemical denaturation in the stomach. Intrinsic factor (IF) is also secreted by parietal cells in the stomach, but its binding to vitamin B12 is weak in the presence of gastric and salivary R-protein. (3) Then, vitamin B12 leaves the stomach and enters the duodenum bound to R-protein and accompanied by IF and by vitamin B12-R-protein complex that have been secreted from the bile. (4) In the mildly alkaline environment of the jejunum, pancreatic enzymes degrade both biliary and dietary vitamin B12-R-protien complexes, releasing free vitamin B12. IF is resistant to proteolysis and binds to the released vitamin B12 to facilitate its active absorption in the ileum. (5) The IF-B12 complex is carried down until the distal 80 cm of the ileum where it binds to mucosal cell receptors (cubilin), then vitamin B12 is bound to transport proteins known as transcobalamin I, II and III (TCI, TCII and TCIII). Vitamin B12 is subsequently transported systemically via the portal system. (6) Within each cell, the TCII–vitamin B12 complex is taken up by means of endocytosis and vitamin B12 is liberated and then converted enzymatically into its two coenzyme forms, methylcobalamin and adenosylcobalamin [from Andres et al. 2004].

After being absorbed in the terminal ileum, B12 is forms a complex with transcobalamin II (TC-II, the active extracellular carrier protein) and is distributed to the tissues by the TC-II receptor-mediate endocytosis. Although newly absorbed B12 is associated with TC-II in a complex known as holo-transcobalamin (holo-TC), 75% of B12 in plasma is actually bound to haptocorrin (or TC-I) (Shane 2008). TC-II levels are 3 to 3 fold higher in plasma than TC-I but the latter is over 90% saturated with cobalamin, compared to about 10% for TC-II. The functional significance of haptocorrin in the human body is not entirely clear but it is believed to facilitate uptake of B12 into the liver, which has receptors for both TC-I and TC-II (Shane 2008).

B12 is predominantly stored in the liver at concentrations of 2 to 4mg, in the form of 5'-deoxyadenosylcobalamin (Shane 2008). So, based on a recommended daily nutrient intake (RNI) of 2mcg/day (it varies from 1.5mcg/day in the UK to 4.5 mcg/day in Europe), it would take 3 to 4 years for deficiency to become apparent (COMA (Committee on Medical Aspects of Food Policy) 1991; EFSA NDA Panel (EFSA Panel on Dietetic Products 2015).

B12 can only be synthesised by certain strains of bacteria (not humans or animals) and animals obtain this vitamin by consuming food contaminated by these bacteria and then incorporating it into their organs. Therefore humans get their B12 by eating foods from animal sources that are rich in B12. These include red meat (2-10mcg/100g, however liver contains up to 95mcg/100g), fish (2-8mcg/100g), eggs (2mcg/100g), milk (1.5mcg/100g) and cheese (1.5mcg/100g) (W. Herrmann and Obeid 2012; J. M. Scott 1997). The bioavailability of dietary B12 is around 50% on average but it varies according to the type and B12 content of food consumed. For instance only around 11% is absorbed from liver, which has a high B12 content (Allen 2008). The quoted figures are 50% absorption from an intake of 1mcg dose, 25% from 5mcg and 5% from 25mcg (Adams et al. 1971). However the maximum absorption possible from a meal is around 3mcg, due to saturation of the intestinal receptors, so any additional absorption is from the 1% which occurs passively through the intestines (W. Herrmann and Obeid 2012). Therefore B12 deficient individuals are often given very high doses of the crystalline form (around 500mcg/day to meet their daily requirements).

### 3.1.3. Biochemical markers of B12 insufficiency

The biomarkers used to diagnose B12 insufficiency in human plasma/serum are summarised in Table 3.1. These include:-

1) Total serum cobalamin: this is usually the first line test done when B12 deficiency is suspected. However, problems with it are that since around 90% of B12 is bound to the inactive haptocorrin, minor changes in the 'active' cobalamin will not be reflected by total cobalamin measurement. This test has low sensitivity and specificity to detect 'true' cobalamin deficiency especially when the readings are between 150 to 300 pmol/l (R. Green 1995; W. Herrmann and Obeid 2012).
2) Holotranscobalamin (holoTC): this is the earliest marker of cobalamin deficiency in the blood (Bor et al. 2004) but drugs like the oral contraceptive pill can lower the values and renal failure increase it.
3) Hcy: this is a useful reflection of functional cobalamin insufficiency but elevation may also be caused by folate or vitamin B6 deficiencies

4) MMA: concentrations above 300nmol/L are the most sensitive and specific marker of cobalamin deficiency (W. Herrmann and Obeid 2012). However false positives can occur with renal insufficiency and intestinal bacterial overgrowth

**Table 3.1** Available markers of vitamin B12 deficiency (from Herrmann and Obeid 2012)

| Parameter | Reference range | Material | Methods | Pre-analytical | Confounding factors |
|---|---|---|---|---|---|
| tHcy | < 12 µM | EDTA plasma, serum | GCMS, HPLC, (EIA) enzymatic immuno-assay, LC-MS/MS | Centrifuge and separate within 45 min preferably keep the sample on ice<br>Several stabilizing agents are currently available (DZA) | Renal function (↑)<br>Age (↑)<br>Medications (↑)<br>Hormone therapy (↑→) |
| MMA | < 0.300 µM | EDTA plasma, serum | GCMS<br>LC_MS/MS | – | Renal function (↑)<br>Age (↑) |
| Cobalamin | > 156 pmol/L | Serum | Chemiiluminescience, Microbiological assay | – | Renal function (↑) |
| holoTC | > 40 pmol/L | EDTA plasma, serum | RIA, ELISA | – | Renal function (↑) |

tHcy: total homocysteine, MMA: methylmalonic acid, holoTC: holotranscobalamin, GCMS: gas chromatograph mass spectrometer , HPLC: high performance liquid chromatography, LC_MS/MS: liquid chromatography mass spectrometry, RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbant assay,

### 3.1.4. Causes, symptoms and diagnosis of B12 insufficiency

The causes of B12 insufficiency in adults are summarised in Table 3.2. Population surveys in Western countries quote B12 insufficiency rates to be 3-5% in adults in their 3$^{rd}$ to 5$^{th}$ decades but it rises to between 6 – 20% in the over 60's in the USA and around 10% of the over 75's in the UK, depending on the cut-off levels used (Allen 2009; Clarke et al. 2004; Pfeiffer et al. 2007). In developing countries, the estimates are 40 to 70% of all adults (Allen 2004; Refsum et al. 2001). The main reason for high rates of insufficiency in the elderly is malabsorption of food-based B12 due to age-related gastric atrophy. Gastric acid is required for the release of B12 from food and subsequent binding to R-protein or haptocorrin. However, absorption of synthetic vitamins from fortified foods or supplements is not affected because they exist in the crystalline form and therefore do not require release in an acidic pH prior to binding to IF.

**Table 3.2** Acquired causes of vitamin B12 deficiency [adapted from Herrmann and Obeid 2012].

| Disease or condition | Mechanism |
|---|---|
| **High risk populations:** vegetarians/vegans and their children, poverty, malnutrition, anorexia nervosa, alcoholics, elderly | Restricted intake of B12 |
| **Pregnancy, lactation** | Increased demands |
| **Medications:**<br>• H2 receptor antagonists, proton pump inhibitors<br>• Oral contraceptive pill<br>• Metformin | <br>• Changing gastrointestinal pH<br>• Decrease in transcobalamin-II<br>• Inhibit B12 absorption |
| **Autoimmune conditions**<br>• Pernicious anaemia (Type A atrophic gastritis)<br>• Age-related atrophic gastritis or H. pylori infection | <br>• Anti-parietal cell/IF antibodies, lack of IF<br>• Change of pH |
| **Gastrointestinal morbidities**<br>• Terminal ileal diseases, pancreatic insufficiency ileal or gastric resection, celiac disease, tropical sprue, Crohn's disease<br>• Small bowel bacterial overgrowth, blind loop syndrome | <br>• Interact with intestinal absorption<br>• Bacteria compete for and break down IF-B12 complex |

A classical finding in B12 or folate insufficiency is megaloblastic anaemia which arises due to double-stranded DNA breaks in a rapidly dividing red blood cell. The defective DNA synthesis occurs due to decreased uracil to thymidylate conversion (as described above) resulting in uracil misincorporation into DNA. Megaloblastosis identified as erythrocytes with a large mean corpuscular volume (MCV) (i.e. macrocytic). However, this can be a late feature of B12 insufficiency and if there is co-existing iron deficiency (which causes microcytosis), the MCV may be normal.

Neurological signs of B12 deficiency include sensory disturbances in the extremities, memory loss and dementia and in infants/children can cause significant developmental regression and poor intellectual outcomes. The reasons for these effects is not entirely clear but are believed to be either due to impaired methylation of essential amino acids or neurotransmitters from methionine synthase deficiency (Weir et al. 1988) or because of build-up of mitochondrial methylmalonyl CoA and propionyl CoA which abnormally accumulate in myelin sheaths causing demyelination (Metz 1992).

From Figure 3.2, it can be seen that the defective DNA synthesis causing megaloblastosis occurs primarily due to deficiency of non-methylated tetrahydrofolate. However it also occurs in B12 deficiency due to the folate trap hypothesis (i.e. folate is trapped as 5-methylfolate because of inhibition of methionine synthase activity). These biochemical mechanisms explain why giving high doses of folate can reverse the haematological abnormalities of B12 deficiency but will not prevent, or may even exacerbate, the neurological complications.

### 3.2. Role of B12 in embryogenesis and pregnancy outcomes

Vitamin B12 and folate vitamins are pivotal in normal embryogenesis and therefore there is increasing attention on optimising levels in young women in the periconceptional period and pregnancy. The levels of both these micronutrients have been associated with pregnancy complications such as neural tube defects (NTD), spontaneous abortion (George et al. 2002), pre-eclampsia (Mujawar et al. 2011; Sanchez et al. 2001) and preterm birth (Ronnenberg et al. 2002), with the latter two conditions mediated in part by elevated Hcy.

NTDs occur when the embryo's neural tube fails to close by around 28$^{th}$ day after conception, causing damage to the underlying neural tissues which is exposed. The resulting defects cause much morbidity and mortality to the developing embryo with the most severe being anencephaly, a condition which is incompatible with life. In spina bifida where the defect is more caudal, several morbidities such as urinary and fecal incontinence and paralysis of the lower limbs occur. The precise reason why folate (and/or B12 deficiency) causes NTDs is not entirely clear but is believed to be related to its interference with cellular proliferation by inhibiting the one-carbon metabolism, DNA synthesis and methylation pathways (Crider et al. 2011; Pitkin 2007). Indeed genetic studies looking at polymorphisms of the MTHFR and other candidate genes causing low folate confirmed that its deficiency was indeed causative for NTDs (Molloy et al. 2009). Randomised control trials have demonstrated a 70 – 100% decrease in NTDs following folic acid supplementation (Czeizel and Dudas 1992; MRC Vitamin Study Research Group 1991). However, the importance of B12

in preventing NTDs cannot be overlooked since it can cause functional folate deficiency (by 'folate trap hypothesis') and is also required for the synthesis of methyl donors.

The reason that B12 (along with folate) is so essential to embryogenesis can be explained by revisiting its role in the one-carbon metabolism and methylation pathways discussed in Section 3.1.1. There are 2 critical periods of DNA methylation reprogramming during early fetal development: firstly during germ cell differentiation (pre-implantation) when rapid genome wide demethylation occurs to reset the genes followed by remethylation to allow parent-specific allele expression at around 12 days post-fertilisation (Santos and Dean 2004) (Figure 3.5). The second phase occurs in the blastocyst stage (days 5-7 post-fertilisation) when de novo methylation begins. Any insults which occur during these periods can result in heritable changes in gene expression that are not due to alterations in DNA (known as epigenetics) (Canovas and Ross 2016; Waterland and Jirtle 2004).

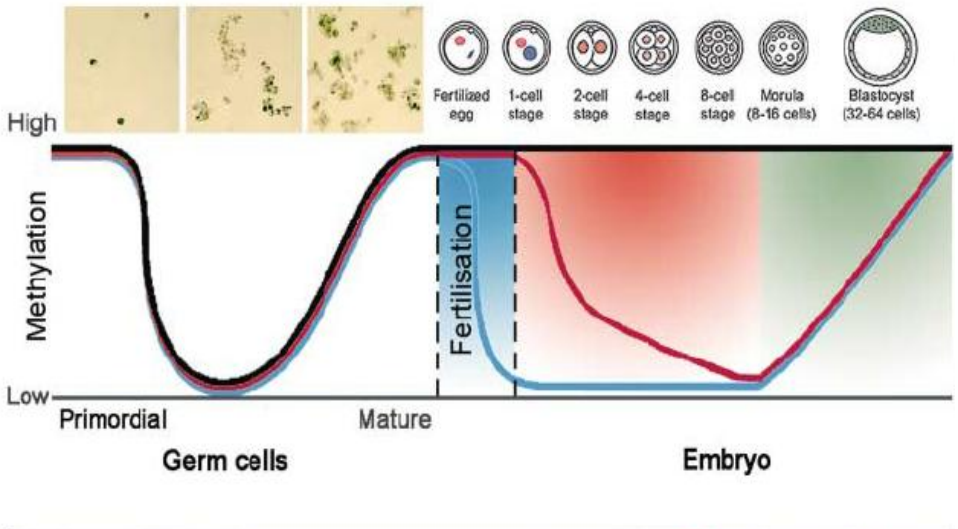


**Figure 3.5** Methylation reprogramming during mouse development. The diagram depicts methylation levels of various classes of genes during germ cell and embryonic development (methylated imprinted genes (black line) and non-imprinted genetic sequences (red, maternal; blue, paternal)). Highly methylated primordial germ cells enter the germinal ridge and undergo loss and reacquisition of methylation during their expansion phase. Examples of these cells (day 11.5, 13.5 and 14.5) stained for alkaline phosphatase, a PGC marker, are pictured above. [from Santos and Dean 2004]

Aside from DNA methylation, other processes that can lead to epigenetic alterations are post-translational modification of histones and noncoding RNAs. Any perturbations during these key points of epigenetic remodelling can lead to permanent

and inheritable changes to the epigenome, which can make the individual susceptible to chronic disease in later life (Figure 3.6) (Ross and Canovas 2016; Warner and Ozanne 2010).

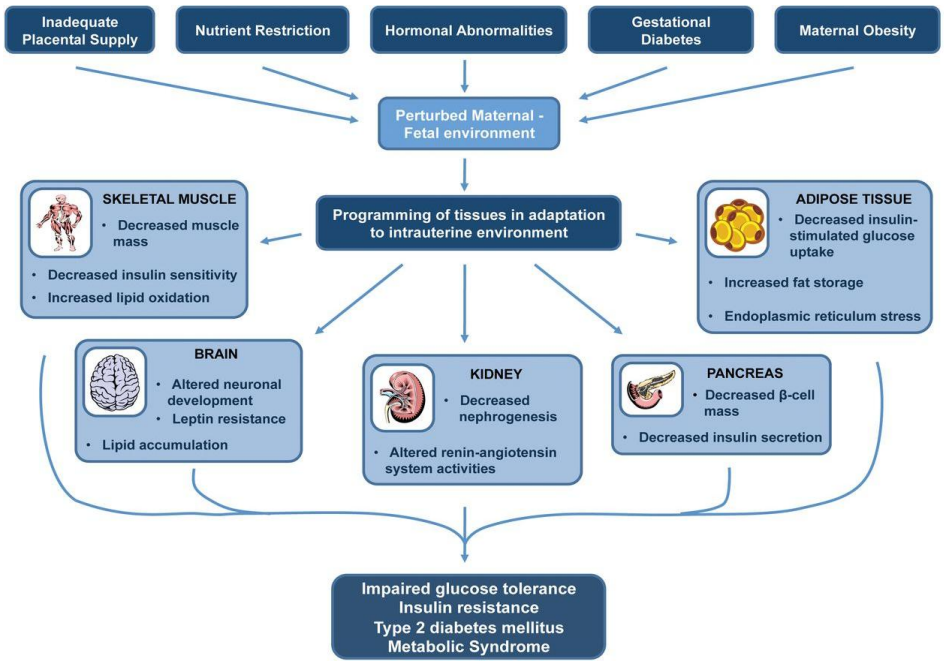


**Figure 3.6** Summary of the structural and molecular adaptations made during the intrauterine programming of metabolic disease. A schematic representation of how a perturbed intrauterine environment, induced by a variety of physiological disturbances in animal models, can elicit changes in the structure and function of multiple organs, subsequently leading to the development of features of metabolic syndrome [from Warner and Ozanne 2010]

Due to the rapid differentiation of the developing embryo, these DNA methylation sequences must be maintained over several cycles of cellular proliferation during the in-utero stage necessitating adequate provision of substrate. Several of the methyl donors required for normal DNA synthesis and methylation are obtained from the diet (e.g. methionine, choline, B12, folic acid, choline) so there is increasing attention being given to optimising the nutritional status of women in the peri-conception period.

The importance of adequate nutrition during pregnancy, especially a methyl-rich diet, has been demonstrated in several animal studies. Maternal calorie restriction leads to lower offspring birthweight with reduced pancreatic beta-cell mass, putting the offspring at increased risk of glucose intolerance, hyperinsulinaemia, hyperleptinaemia and obesity later on (Bertin et al. 2002; Garofano et al. 1997;

Vickers et al. 2000). Similar metabolic outcomes were also observed in lambs born to sheep fed a 'methyl-deficient diet' during pregnancy, highlighting the key role of the intra-uterine environment in determining future metabolic diseases (Sinclair et al. 2007).

### 3.3. Next steps

In the next 3 chapters I will examine, in two systematic reviews, the prevalence of vitamin B12 insufficiency in pregnancy across the world and its impact on fetal birth weight followed by a retrospective case-control study of scale of the problem in a UK population of pregnant women with and without GDM. Finally I will discuss if there needs to be a national policy change with respect to dietary reference intake for B12 by utilising micronutrient intake and biomarker results from the National Dietary Nutrition Survey in women of child-bearing age.

# Chapter 4

# Prevalence of vitamin B12 insufficiency during pregnancy and its effect on offspring birthweight: a systematic review and meta-analysis

## 4.1. Introduction

In Chapter 3, I have discussed the role that vitamin B12 (B12) plays in normal embryogenesis and potential impact of insufficiency during pregnancy.

Low B12 can have an impact on fetal birthweight by influencing placental development (Koukoura et al. 2012) but the relationship between B12 and birth weight is far from established (Furness et al. 2013; Hogeveen et al. 2010). LBW or small for gestational age (SGA) are outcomes of particular interest as they are well-established surrogate markers for metabolic disorders such as obesity, type 2 diabetes and metabolic syndrome in later life in many populations (Hales and Barker 2001; Whincup et al. 2008; C.S. Yajnik et al. 1995). Most studies showing the link between B12 and LBW are from Indian subcontinent, where rates of both LBW/SGA and B12 insufficiency are high (Muthayya et al. 2006b; United Nations Children's Fund and World Health Organization 2004; C. S. Yajnik et al. 2008). High prevalence of B12 insufficiency in India has been attributed to vegetarianism (i.e. no consumption of animal products except for dairy) and infrequent meat consumption in omnivores (i.e. consumption of small quantities of non-vegetarian food less often than alternate days) (Refsum et al. 2001; C. S. Yajnik et al. 2008). However, B12 insufficiency has also been found in other countries where vegetarianism is rare such as Brazil and Turkey (Ackurt et al. 1995; Guerra-Shinohara et al. 2004).

The aims of this systematic review and meta-analysis are to evaluate the prevalence of vitamin B12 (B12) insufficiency in pregnancy across a worldwide population and assess whether this is associated with low birthweight (LBW) and/or small for gestational age (SGA).

## 4.2. Methods

This systematic review comprises of 2 sub-reviews (namely (1) Prevalence of B12 insufficiency and (2) B12 insufficiency and birthweight (BW)) and in the following sections will be divided accordingly where appropriate.

### 4.2.1. Sources of data

Published guidelines on reporting systematic reviews and meta-analysis of observational studies (MOOSE Guidelines) were followed (Stroup et al. 2000). A

comprehensive literature search in 5 bibliographic databases was conducted for the 'Prevalence sub-review': MEDLINE/PUBMED (National Library of Medicine and National Institute of Health), EMBASE (The Excerpta Medica database), Global health (CABI), CAB (Commonwealth Agricultural Bureau database) and CINAHL (The Nursing & Allied Health database). For the 'Birthweight sub-review', 4 databases were used, namely MEDLINE/PUBMED, EMBASE, Global Health and Scopus (Elsevier). All databases were searched from inception until December 2014. We also examined reference lists of key publications for further articles. Where needed, the authors were contacted by email for more complete information.

#### 4.2.2. Search criteria

For the 'Prevalence sub-review', a search strategy based on the following keywords and medical subject headings (MeSH) was used: "cobalamin", "vitamin B12", "vitamin B12 insufficiency", "vitamin B12 deficiency", "methylmalonic acid", "holotranscobalamin", "homocysteine", "pregnancy", "pregnant women". Search words were combined using Boolean operators (AND, OR). A similar search string was used in all bibliographic databases. Studies conducted on adult pregnant women (aged 18-45 years) at any trimester, including delivery, which report prevalence of vitamin B12 insufficiency with clearly defined cut-off levels were included. Only results from the trimesters where B12 values were available from at least 50 women are reported.

The keywords and MeSH used for the 'Birthweight sub-review' included: "cobalamin", "vitamin B12 insufficiency", "vitamin B12 deficiency", "methylmalonic acid", "holotranscobalamin", "homocysteine", "pregnancy outcome", "birth weight", "intrauterine growth retardation", "small for gestational age". An identical approach to that described above was used for combining search words.

#### 4.2.3. Eligibility criteria

Both longitudinal and cross-sectional observational studies, conducted in the community or hospital setting on adult pregnant women (aged 18 to 45 years) without any major co-morbidities were included. We restricted the search to studies conducted on human subjects and published in English language in peer-reviewed journals. If the results of a study were reported in more than one publication, the study with the most

complete information pertaining to our review's outcomes was used. If these were identical, the one published earlier was included. The following types of studies were excluded: randomised controlled trials where B12 supplementation was given as part of the study design, case-control studies and studies done exclusively on mothers with co-morbidities (e.g. HIV, post-bariatric surgery). Additionally, we excluded studies which were designed ***specifically*** to look at pregnancies with neural tube defects, intra-uterine growth retardation, early pregnancy loss and anaemia (however, if the studies were conducted on 'normal' pregnant women with no prior medical history and reported rates of anaemia in their results, they were included). For the 'Birthweight sub-review', we included studies which reported vitamin B12 results from maternal or cord blood and offspring birth weight.

#### 4.2.4. Data extraction

Level one screening of initial database search results (titles and abstracts) was independently performed by at least two reviewers (NS and NB/SBR) according to the inclusion and exclusion criteria. Level two screening was conducted by reviewing the full manuscripts of the articles. Two reviewers (NS and SBR) independently extracted the study characteristics onto pre-designed forms which included information on the study population and methods, vitamin B12 values and birthweight outcomes. Any discrepancy in data extraction was resolved by consensus and consulting a third reviewer (PS) where necessary.

#### 4.2.5. Data synthesis

For the 'Prevalence sub-review', we analyzed and reported the results of the systematic review according to the 3 trimesters of pregnancy, to ensure like-for-like comparison. It is well known from longitudinal studies that there is a progressive decline of B12 during the course of pregnancy (Cikot et al. 2001; Garcia-Casal et al. 2005), which reaches a nadir towards the end of the 3$^{rd}$ trimester (Van Sande et al. 2013). To assess the impact of geography on the worldwide prevalence of B12 insufficiency, the broad World Health Organization (WHO) Region classification was used (i.e. Africa, Americas, South-east Asia, Europe, Eastern Mediterranean and Western Pacific) (McLean et al. 2008). However, we further divided the Americas and Southeast Asia regions into North America and Central/South America and Indian sub-continent and South-east/East Asia respectively in an attempt to bring

together the populations based on dietary habits, vegetarianism and consumption of animal products (Stabler and Allen 2004).

For the 'Birthweight sub-review', the included studies reported three different types of effect sizes, namely 1) odds of having an adverse birthweight outcome below a threshold of maternal/cord B12, 2) comparison of mean/median maternal/cord B12 values between adverse and normal birthweight, and 3) the effect of maternal/cord B12 as a linear variable on birthweight (regression coefficient or correlation coefficient). Adverse birth weight outcome was defined as LBW (birthweight <2500g) (United Nations Children's Fund and World Health Organization 2004), SGA (birthweight <$10^{th}$ centile for gestational age), intra-uterine growth restriction (IUGR) (estimated fetal weight <10%) (Goldenberg and Cliver 1997) or as the lowest tertile or quartile of BW in the included studies.

### 4.2.6. Statistical analysis

*Prevalence sub-review*

To estimate the pooled estimates of the B12 insufficiency rate per trimester, we obtained an estimate from each study of the proportion of pregnant women with B12 below the cut-off level defined in that study. Sub-group analysis was undertaken for the studies from the $2^{nd}$ and $3^{rd}$ trimesters to determine if the prevalence of B12 insufficiency in pregnancy varied according the geographical areas. Statistical heterogeneity was calculated using the $I^2$ statistic (Higgins and Green 2011). A random effects meta-analysis was undertaken using STATA version 13 software (DerSimonian and Laird 1986; StataCorp 2011). We assessed publication bias by using a funnel plot, Egger's and Begg's tests to find out whether there was a bias towards publication of studies with positive results among the smaller studies (results not shown).

In order to correct for differences in the vitamin B12 measurement assays and cut-off levels used by the studies, we calculated a standardised score by dividing the mean B12 level used in the study by the cut-off level used to define insufficiency in that study (mean B12 ÷ insufficiency cut-off). In the studies where a median B12 value was reported, it was used to estimate the mean where sample sizes were large (Hozo et al. 2005). Stepwise linear regression was then done to determine the predictors of

percentage B12 insufficiency in a model which included the trimester of sampling, assay type and geographical region. Log-transformed standardised scores were used for this as the variable was not normally distributed. SPSS version 22 was used for the analysis (IBM Corp Released 2013).

*Birthweight sub-review*

The software Review Manager (2013) was used to conduct meta-analyses from the included studies. We obtained an estimate from each study of the adjusted odds ratio (OR) with 95% confidence intervals (CIs) using a random effects model. Statistical heterogeneity was calculated using the $I^2$ statistic (Higgins and Green 2011). Adjusted outcome measures were tabulated where these were reported. Continuous effect measures data were expressed as mean difference (SD) of B12 between babies of low birthweight or the equivalent (termed as 'adverse birthweight outcome cases') and normal birthweight outcome. Pooled analyses were not done for the studies reporting B12 and BW as linear variables because there was too much heterogenity in the reporting of the independent variable and outcome (e.g. some reporting unit values and others standard deviation scores).

### 4.2.7. Risk of bias and quality of evidence

The methodological quality assessment of the studies was done using a checklist for cohort studies adapted from the Scottish Intercollegiate Guidelines Network (Scottish Intercollegiate Guidelines Network (SIGN) and Healthcare Improvement Scotland 2011). The quality assessment focussed on evaluating how minimal the risk of bias was in study reporting using 14 key criteria from the checklist (Appendix 1.1). However, for certain cross-sectional studies that reported only the point prevalence of B12 insufficiency, only 12 relevant criteria were used (criteria relating to drop out rate and comparison between full participants and those lost to follow-up were not used, as these were not relevant). The overall study quality grade was calculated as per standard guidelines according to the proportion of total criteria fulfilled (Scottish Intercollegiate Guidelines Network (SIGN) and Healthcare Improvement Scotland 2011).

## 4.3. Results

### 4.3.1. Prevalence of B12 insufficiency sub-review

*Study characteristics*

The electronic database search yielded 4742 citations, of which 153 were selected for full-text review (Figure 4.1A). There were six studies identified from the full text review, which reported B12 insufficiency rates during pregnancy but without specifying a trimester (Ball and Giles 1964; Bondevik et al. 2001; Ipciglu et al. 2007; D. J. Vanderjagt et al. 2007) or clearly stating the number of women sampled per trimester (Brabin et al. 1986; Haliloglu et al. 2010). These studies were not included in further analysis.

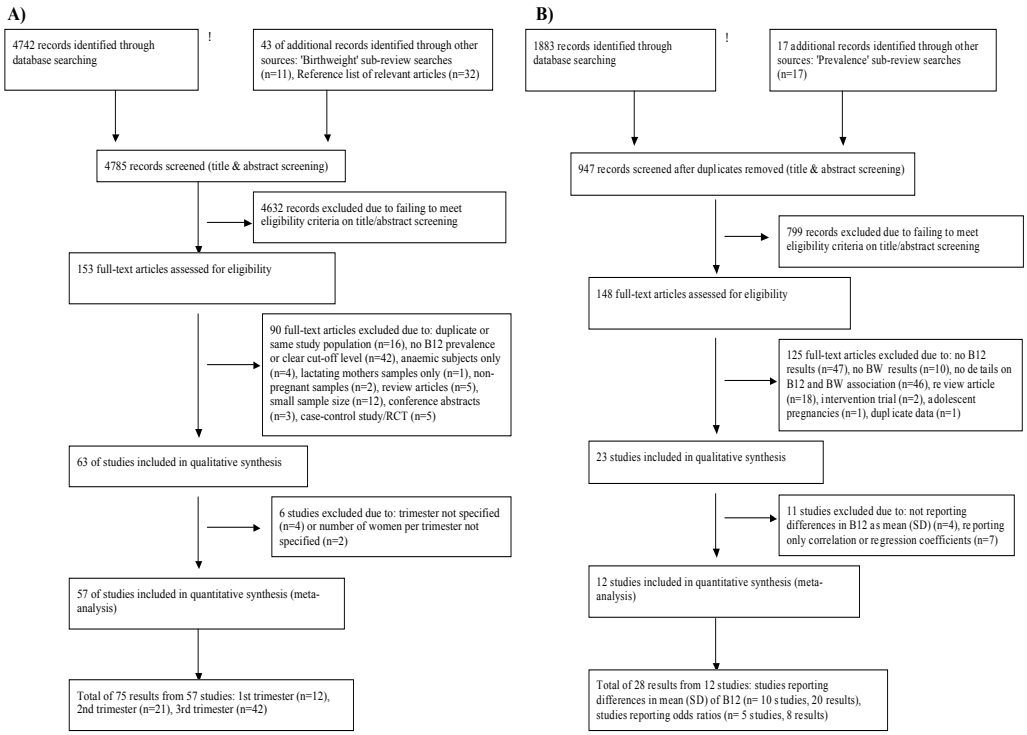


**Figure 4.1** PRISMA flow diagram showing the study selection process.
**A)** Prevalence of B12 insufficiency in pregnancy sub-review and **B)** B12 insufficiency in pregnancy and birth weight sub-review

A total of 57 studies met all the inclusion criteria, comprising of 16 longitudinal studies (n=34 results) and 41 cross-sectional studies, giving a total of 75 results (Abdelrahim et al. 2009; Ackurt et al. 1995; Areekul et al. 1976; H Baker et al. 1975; Balci et al. 2014; Barbosa et al. 2008; Bjorke Monsen et al. 2001; A. K. Black et al.

1994; Bruinse and van den Berg 1995; Cole et al. 1974; Colman et al. 1975; Cook et al. 1971; Dwarkanath et al. 2013; Frery et al. 1992; Garcia-Casal et al. 2005; Gibson et al. 2008; Giugliani et al. 1984; Goedhart et al. 2011; Guerra-Shinohara et al. 2004; Halicioglu et al. 2012; Hall et al. 2007; Heppe et al. 2013; Hinderaker et al. 2002; C. H. Ho et al. 1987; House et al. 2000; Hussein et al. 2009; Jacob et al. 1976; Jacquemyn et al. 2013; Jiang et al. 2005; Katre et al. 2010; E. M. Knight et al. 1991; Koc et al. 2006; Kosus et al. 2012; Krishnaveni et al. 2009; Li et al. 2008; Lowenstein et al. 1960; Ma et al. 2004; Marzan et al. 1971; Milman et al. 2006; Murphy et al. 2007; Osifo and Onifade 1976; Pagán et al. 2002; Park et al. 2004; Pathak et al. 2007; Ray et al. 2008; Roberts et al. 1973; Samuel et al. 2013; Schulpis et al. 2004; Shamim et al. 2013; Shields et al. 2011; Takimoto et al. 2007; D. Vanderjagt et al. 2009; Whiteside et al. 1968; Wu et al. 2013; C. S. Yajnik et al. 2008; Yusufji et al. 1973; Zachau-Christiansen et al. 1962). Details of these studies including the country where the field work was done, proportion of B12 supplement or multivitamin use, B12 assay method and insufficiency rates are presented in Table 4.1. For the setting of the study, they were broadly categorised into a community or hospital setting (including health centres) to reflect where the population was sampled from. In the first, second and third trimesters, there were 12, 21 and 42 results, which were obtained by sampling 10474, 8621 and 11667 pregnant women respectively.

**Table 4.1** Prevalence of B12 insufficiency during pregnancy: Key study characteristics and results

| **Author, Year of publication Country, Year of field study** | **Study design[1] No. of participants with B12 results** | **Setting of Study Population response rate** | **B12 supplement or multivitamin use (%)[2]** | **B12 cut-off level (pmol/l)** | **% B12 insufficiency** | **Mean B12 level, pmol/l (S.D.)** |
|---|---|---|---|---|---|---|
| **1st trimester (n=12)** | | | | | | |
| **a) Microbiological assay** | | | | | | |
| **Whiteside et al. 1968 (a)** Australia, N/R | L 56 | Hospital N/R | N/R | <74 | 5 | 217 |
| **Roberts et al. 1973 (a)** England, 1971 | L 320 | Hospital N/R | N/R | <118 | 35 | 165 (84.9) |
| **Jiang et al. 2005** Nepal, 1998-2001 | CS 1158 | Community 89% consented | None | <150 | 28.3 | 237.1 (138.3) |
| **Murphy et al. 2007 (a)** Spain, 1992-96 | L 88 | Hospital N/R | 27%[2] | <150 | 0 | 267 (144, 449)[3] |
| **b) Radioimmunoassay** | | | | | | |
| **Garcia-Casal et al. 2005 (a)** Venezuela, 2001-02 | CS 129 | Hospital N/R | N/R | <148 | 43.4 | N/R |
| **Ray et al. 2008** Canada, 2007 | CS 3734 | N/R N/R | N/R | <125 | 8.5 | 249 (244, 255)[4] |
| **c) Chemiluminescence** | | | | | | |
| **Kosus et al. 2012** Turkey, N/R | CS 228 | Hospital N/R | N/R | <156 | 12.5 | 200 (95.6)[5] |
| **Dwarkanath et al. 2013** India, N/R | L 1838 | Hospital 73% consented | None | <150 | 32 | N/R |
| **Heppe et al. 2013** Netherlands, 2002-06 | CS 2173 | Hospital N/R | N/R | <150 | 26 | 175 (100)[5] |
| **Samuel et al. 2013** India, 2008-10 | CS 352 | Hospital 88% consented | None | <150 | 51.1 | 149 (109, 205)[5] |
| **Shamim et al. 2013** Bangladesh, 2001-07 | CS 285 | Community N/R | N/R | <150 | 19.6 | 206.3 (84.5) |
| **d) Assay method not described** | | | | | | |
| **Shields et al. 2011 (a)** Scotland, 2008-09 | CS 113 | Hospital N/R | N/R | N/R <156 | 16 | 215 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **2nd trimester (n=21)** | | | | | | |
| **a) Microbiological assay** | | | | | | |
| **Lowenstein et al. 1960 (a)**<br>Canada, N/R | L<br>59 | N/R<br>N/R | N/R | <148 | 15.2 | 235 (101) |
| **Whiteside et al. 1968 (b)**<br>Australia, N/R | L<br>50 | Hospital<br>N/R | N/R | <74 | 25 | 127 |
| **Jacob et al. 1976**<br>USA, 1972-74 | CS<br>182 | Hospital<br>100% consented | 20% | <111 | 4.5 | 303 |
| **Murphy et al. 2007 (b)**<br>Spain, 1992-96 | L<br>90 | Hospital<br>N/R | 27%[2] | <150 | 0 | 230 (123, 432)[3] |
| **Yajnik et al. 2008 (a)**<br>India, 1994-96 | L<br>638 | Community<br>92% consented | N/R | <150 | 60 | 135 (103, 175)[5] |
| **Katre et al. 2010**<br>India, 2004-06 | L<br>163 | Hospital<br>97.3% consented | 29%[2] | <150 | 73 | 119 (87, 161)[5] |
| **b) Radioimmunoassay** | | | | | | |
| **Marzan et al. 1971 (a)**<br>Philippines, N/R | CS<br>100 | Hospital<br>N/R | None | <59 | 1.5 | 270.1 (79) |
| **Areekul et al. 1976 (a)**<br>Thailand, N/R | CS<br>71 | Hospital<br>N/R | N/R | <111 | 7 | *All trimesters:* 211 (106) |
| **Knight et al. 1991 (a)**<br>USA, 1985-90 | L<br>108 | Hospital<br>N/R | 91%[2] | <148 | 7 | N/R |
| **Bruinse et al. 1995 (a)**<br>Netherlands, N/R | L<br>70 | Hospital<br>N/R | None | <180 | 0 | N/R |
| **Ackurt et al. 1995 (a)**<br>Turkey, 1991 | L<br>129 | Hospital<br>66% responded to invitation | 35% | <111 | 48.8 | 140.8 (105) |
| **Pagan et al. 2002 (a)**<br>USA, 1986-88 | L<br>285 | N/R<br>N/R | N/R | <148 | 0.35 | 357 (131) |
| **Park et al. 2004**<br>South Korea, | CS<br>89 | Hospital<br>N/R | 42% | <258 | 46.1 | N/R |
| **Garcia-Casal et al. 2005 (b)**<br>Venezuela, 2001-02 | CS<br>430 | Hospital<br>N/R | N/R | <148 | 58.6 | N/R |
| **Li et al. 2008**<br>Bangladesh, 2002 | L<br>753 | Community<br>78% consented | N/R | <185 | 60 | N/R |
| **c) Chemiluminescence** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Takimoto et al. 2007 (a)**<br>Japan, 2001-03 | L<br>77 | Hospital<br>N/R | N/R | <148 | 8 | 301 (96) |
| **Goedhart et al. 2011**<br>Netherlands, 2003-04 | CS<br>2921 | Community<br>35% consented | N/R | <148 | 6 | N/R |
| **d) Enzyme immunoassay** | | | | | | |
| **House et al. 2000**<br>Canada, 1996-97 | CS<br>1424 | N/R<br>N/R | N/R | <130 | 25.3 | 180 (130, 240)[5] |
| **Milman et al. 2006 (a)**<br>Denmark, 1995-96 | L<br>406 | N/R<br>N/R | 34%[2] | <150 | 15 | 225 (118, 381)[6] |
| **Wu et al. 2013 (a)**<br>Canada, N/R | L<br>264 | Hospital<br>N/R | N/R | <148 | 10 | 287 (126) |
| **e) Others** | | | | | | |
| **Hinderaker et al. 2002**<br>Tanzania, 1995-96 | CS<br>312 | Hospital<br>78% consented | N/R | HPLC<br><150 | 16.7 | N/R |
| **3rd trimester (n=42)** | | | | | | |
| **a) Microbiological assay** | | | | | | |
| **Lowenstein et al. 1960 (b)**<br>Canada, N/R | L<br>252 | N/R<br>N/R | N/R | <148 | 19 | 221 (126) |
| **Zachau-Christiansen et al. 1962**<br>Denmark, N/R | CS<br>365 | Hospital<br>N/R | N/R | <111 | 17 | 177 |
| **Roberts et al. 1973 (b)**<br>England, 1971 | L<br>119 | Hospital<br>N/R | N/R | <118 | 48 | 134 (70.9) |
| **Yusufji et al. 1973**<br>India, N/R | CS<br>998 | Hospital<br>N/R | N/R | <103 | 52 | 117 (90) |
| **Baker et al. 1975**<br>USA, N/R | CS<br>174 | N/R<br>N/R | 76%[2] | <59 | 23 | 85 (832) |
| **Osifo et al. 1976**<br>Nigeria, N/R | CS<br>50 | N/R<br>N/R | N/R | <148 | 40 | 208 (123) |
| **Bjorke-Monsen et al. 2001**<br>Norway, 1996-97 | CS<br>169 | Hospital<br>N/R | 36%[2] | <150 | 15 | 245 (175, 323)[5] |
| **Murphy et al. 2007 (c)**<br>Spain, 1992-96 | L<br>90 | Hospital<br>N/R | 27%[2] | <150 | 0 | 224 (117, 444)[3] |
| **Pathak et al. 2007**<br>India, N/R | CS<br>266 | Community<br>94% consented | N/R | <148 | 74.1 | N/R |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Yajnik et al. 2008 (b)**<br>India, 1994-96 | L<br>594 | Community<br>92% consented | N/R | <150 | 71 | 122 (94, 160)[5] |
| **Krishnaveni et al. 2009**<br>India, 1997-98 | CS<br>774 | Hospital<br>N/R | 31%[2] | <150 | 43 | 162 (123,221)[5] |
| **b) Radioimmunoassay** | | | | | | |
| **Marzan et al. 1971 (b)**<br>Philippines, N/R | CS<br>57 | Hospital<br>N/R | None | <59 | 0 | 286.3 (86) |
| **Cole et al. 1974**<br>Australia, N/R | CS<br>130 | Hospital<br>N/R | N/R | <148 | 12.3 | 272.3 |
| **Colman et al. 1975**<br>South Africa, N/R | CS<br>106 | Hospital<br>N/R | N/R | <295 | 0.9 | 524 (165) |
| **Areekul et al. 1976 (b)**<br>Thailand, N/R | CS<br>100 | Hospital<br>N/R | N/R | <111 | 13 | *All trimesters:* 211 (106) |
| **Frery et al. 1992**<br>France, N/R | CS<br>188 | Hospital<br>N/R | N/R | <148 | 27.6 | 175 (74, 397)[4] |
| **Giugliani et al. 1984**<br>Brazil, N/R | CS<br>51 | Hospital<br>100% | 51%[2] | <165 | 21.6 | 251 (108) |
| **Ho et al. 1987**<br>Taiwan, N/R | CS<br>221 | Hospital<br>N/R | None | <110 | 3.6 | 228.6 (157.3) |
| **Knight et al. 1991 (b)**<br>USA, 1985-90 | L<br>218 | Hospital<br>N/R | 91%[2] | <148 | 11.2 | 318 (216) |
| **Black et al. 1994**<br>Mexico, 1985-87 | CS<br>85 | Community<br>N/R | N/R | <74 | 15 | 228 (451) |
| **Ackurt et al. 1995 (b)**<br>Turkey, 1991 | L<br>87 | Hospital<br>66% responded to invitation | 35% | <111 | 80.9 | 94.6 (107.8) |
| **Bruinse et al. 1995 (b)**<br>Netherlands, N/R | L<br>70 | Hospital<br>N/R | None | <180 | 0 | N/R |
| **Pagan et al. 2002 (b)**<br>USA, 1986-88 | L<br>285 | N/R<br>N/R | N/R | <148 | 2.1 | 285 (100) |
| **Ma et al. 2004**<br>China, 1999-2000 | CS<br>1019 | Hospital<br>N/R | None | <148 | 10.5 | N/R |
| **Garcia-Casal et al. 2005 (c)**<br>Venezuela, 2001-02 | CS<br>301 | Hospital<br>N/R | N/R | <148 | 68.5 | N/R |
| **Hall et al. 2007** | CS | Hospital | N/R | <185 | 58.9 | 180.0 (71.5) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bangladesh, 2004-05 | 95 | N/R | | | | |
| **Gibson et al. 2008**<br>Ethiopia, N/R | CS<br>83 | Community<br>N/R | N/R | <150 | 23 | 268 (152, 372)[5] |
| **c) Chemiluminescence** | | | | | | |
| **Schulpis et al. 2004**<br>Greece, 1999-2002 | CS<br>1933 | Hospital<br>N/R | None | <170 | 52.7 | N/R |
| **Koc et al. 2006**<br>Turkey, N/R | CS<br>180 | Hospital<br>N/R | 19% | <118 | 72 | 95.9 (45.5) |
| **Takimoto et al. 2007 (b)**<br>Japan, 2001-03 | L<br>82 | Hospital<br>N/R | N/R | <148 | 16 | 265 (95) |
| **Barbosa et al. 2008**<br>Brazil, 2001-03 | CS<br>275 | Hospital<br>N/R | None | <179 | 75 | N/R |
| **Hussein et al. 2009**<br>Egypt, N/R | CS<br>84 | Hospital<br>N/R | N/R | <150 | 46.4 | 185.3 (113) |
| **Vanderjagt et al. 2009**<br>Nigeria, N/R | CS<br>98 | Hospital<br>N/R | None | <148 | 12.2 | 208 (25, 739)[7] |
| **Halicioglu et al. 2012**<br>Turkey, 2008 | CS<br>208 | Hospital<br>88% consented | 57%[2] | <118 | 47.6 | 120 (N/R)[5] |
| **Balci et al. 2014**<br>Turkey, N/R | CS<br>72 | N/R<br>N/R | None | <148 | 70.8 | 120 (53.1) |
| **d) Enzyme immunoassay** | | | | | | |
| **Guerra-Shinoara et al. 2004**<br>Brazil, N/R | CS<br>119 | Hospital<br>N/R | N/R | <132 | 52.9 | 130 (122,138)[4] |
| **Milman et al. 2006 (b)**<br>Denmark, 1995-96 | L<br>256 | N/R<br>N/R | 34%[2] | <150 | 42.6 | 161 (71, 284)[6] |
| **Wu et al. 2013 (b)**<br>Canada, N/R | L<br>220 | Hospital<br>N/R | N/R | <148 | 23 | 224 (96.2) |
| **e) Others** | | | | | | |
| **Abdelrahim et al. 2009**<br>Sudan, 2007-09 | CS<br>55 | Hospital<br>N/R | N/R | Immunofluorescence<br><111 | 1.1 | 159.4 (66.5) |
| **f) Assay method not described** | | | | | | |
| **Cook et al. 1971**<br>Latin America, | CS<br>899 | N/R<br>N/R | N/R | <59 | 15.4 | N/R |
| **Shields et al. 2011 (b)**<br>Scotland, 2008-09 | L<br>77 | Hospital<br>N/R | N/R | <156 | 60 | 153 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Jacquemyn et al. 2014**<br>Belgium, 2011 | CS<br>78 | Hospital<br>N/R | 76%[2] | <150 | 13 | 244 (93.9) |

Table showing the key study characteristics and results from the 57 included studies classified according to the trimesters of pregnancy and vitamin B12 measurement assay. The lower case letters in parenthesis in the first column indicate the order of appearance in the table for studies which have reported results from more than one trimester. N/R: not reported

[1] L= Longitudinal (i.e. the same participants had more than one B12 measurement during pregnancy); CS=Cross-sectional

[2] Study states explicitly that the women consumed B12 supplements or multivitamins containing B12 (all such values in this column)

[3] Geometric mean ($10^{th}$, $90^{th}$ centile)

[4] Geometric mean ($5^{th}$, $95^{th}$ centile)

[5] Median ($25^{th}$, $75^{th}$ centile or IQR)

[6] Median ($5^{th}$, $95^{th}$ centile)

[7] Median (min, max)

*Prevalence of Vitamin B12 insufficiency in pregnancy*

The overall prevalence of maternal vitamin B12 insufficiency during pregnancy from all the studies, across all three trimesters, was 25%. When analysing by trimester, the rates were 21%, 19% and 29% for the 1st, 2nd and 3rd trimesters respectively (Figure 4.2-4.4).

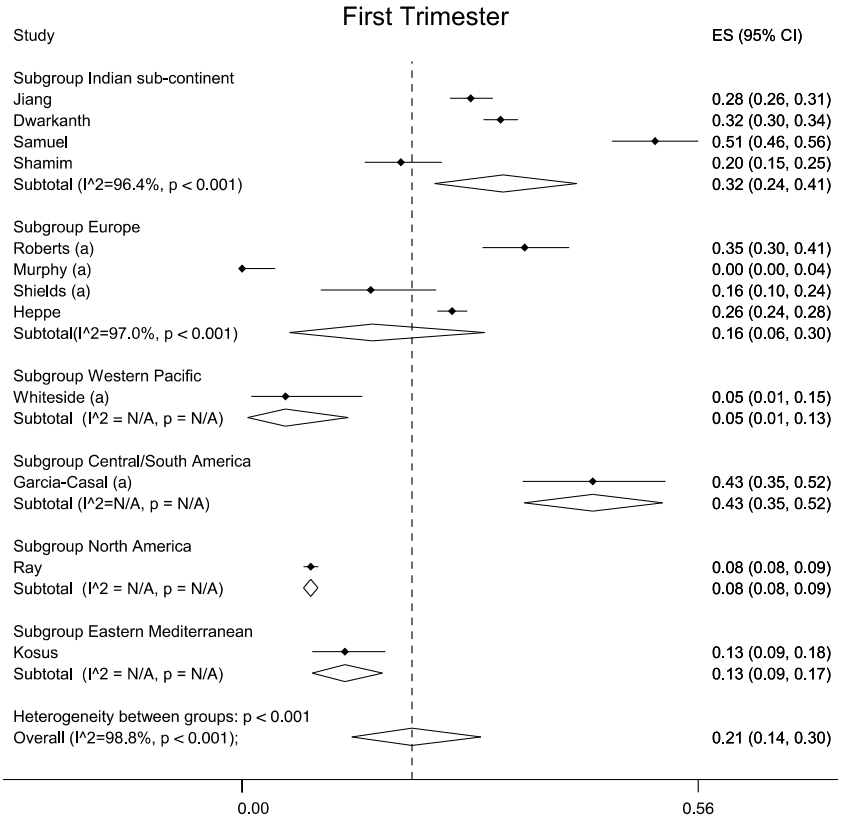


**Figure 4.2** Meta-analysis of maternal B12 insufficiency in the 1st trimester of pregnancy separated by subgroups of geographic regions (n=12 results). The diamonds represent the pooled proportions for each subgroup and the overall proportion for the trimester while the solid diamonds in each study denote the proportion for that study (bars are 95% CI). The $I^2$ values refer to the statistical heterogenity within each subgroup and the whole trimester combined. A random effects model using generic inverse variance showed a pooled proportion insufficiency rate of 0.21 (0.14, 0.30). ES: effect size, N/A: not applicable

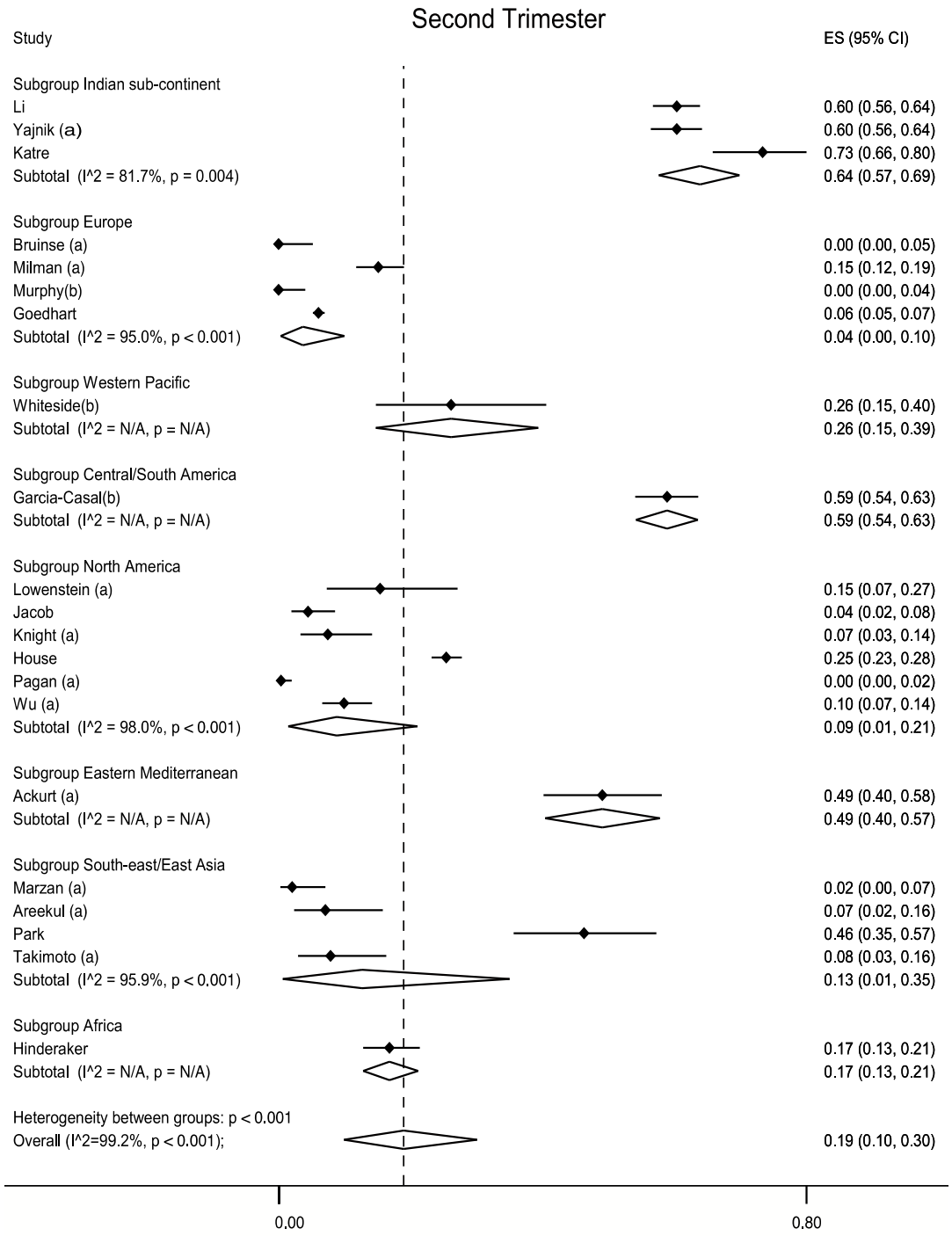


**Figure 4.3** Meta-analysis of maternal B12 insufficiency in the 2nd trimester of pregnancy separated by subgroups of geographic regions (n=21 results). The diamonds represent the pooled proportions for each subgroup and the overall proportion for the trimester while the solid diamonds in each study denote the proportion for that study (bars are 95% CI). The $I^2$ values refer to the statistical heterogenity within each subgroup and the whole trimester combined. A random effects model using generic inverse variance showed a pooled proportion insufficiency rate of 0.19 (0.10, 0.30). ES: effect size, N/A: not applicable

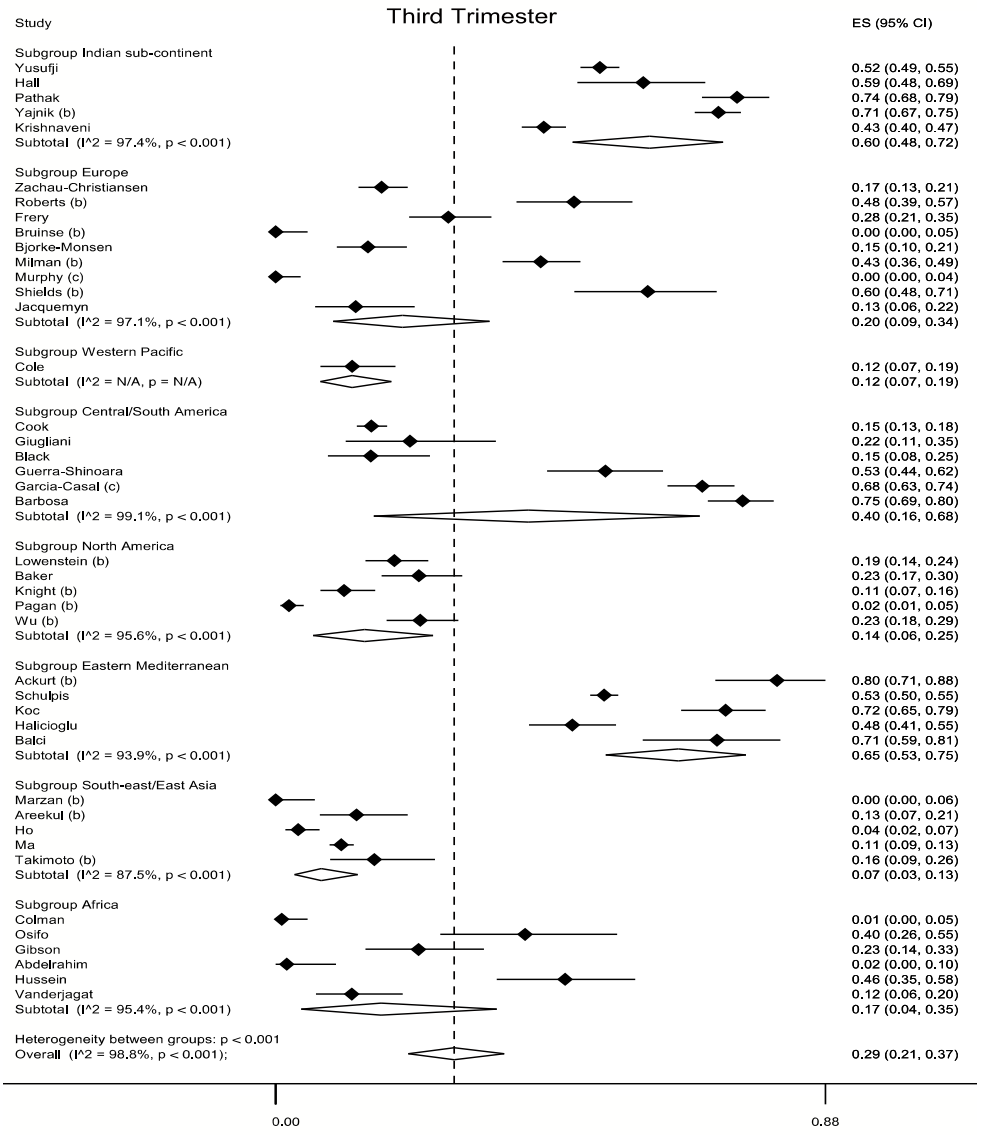


**Figure 4.4** Meta-analysis of maternal B12 insufficiency in the 3rd trimester of pregnancy separated by subgroups of geographic regions (n=42 results). The diamonds represent the pooled proportions for each subgroup and the overall proportion for the trimester while the solid diamonds in each study denote the proportion for that study (bars are 95% CI). The $I^2$ values refer to the statistical heterogenity within each subgroup and the whole trimester combined. A random effects model using generic inverse variance showed a pooled proportion insufficiency rate of 0.29 (0.21, 0.37). ES: effect size, N/A: not applicable

Of the 1st trimester studies, there was insufficient representation from all the geographical regions to do a comprehensive sub-group analysis. The four studies from the Indian sub-continent showed a high insufficiency rate (pooled estimate 32%, Figure 4.2). This observation was once again seen in the 2nd trimester, with the pooled insufficiency rate from the Indian sub-continent rising to 64% (Figure 4.3). Apart from North America, South-east/East Asia and Europe, the other geographical regions

were also poorly represented in the 2nd trimester but there were notably high insufficiency rates of 59%, 49%, 46% found from studies carried out in Venezuela (Garcia-Casal et al. 2005), Turkey (Ackurt et al. 1995) and South Korea (Park et al. 2004).

Of the studies included in the 3rd trimester, the pooled insufficiency rate of the studies from the Indian sub-continent was 60%. An additional striking finding was the pooled insufficiency rate of 65% ($I^2$ 95%) from the five studies in the Eastern Mediterranean region. (Figure 4.4) (Ackurt et al. 1995; Balci et al. 2014; Halicioglu et al. 2012; Koc et al. 2006; Schulpis et al. 2004). On the contrary, rates of <8% insufficiency were found in two studies done in Thailand and Sudan where the authors attributed the low rates of insufficiency to consumption of fish and animal/fermented products respectively (although details of dietary intake were not provided) (Abdelrahim et al. 2009; Areekul et al. 1976).

*Mean vitamin B12 levels across the trimesters*

Eleven studies which we included in this review reported mean B12 results through the course of pregnancy and two studies reported median B12 results longitudinally. Ten of the 11 studies reporting the mean showed a consistent fall in the B12 levels from the 1st to 3rd (Roberts et al. 1973; Shields et al. 2011), 2nd to 3rd (Ackurt et al. 1995; Ipciglu et al. 2007; Lowenstein et al. 1960; Pagán et al. 2002; D. J. Vanderjagt et al. 2007) and from the 1st to 2nd to 3rd trimesters (Murphy et al. 2007; Takimoto et al. 2007; Whiteside et al. 1968). The exception was Marzan et al which showed a marginal rise in the mean B12 levels across the pregnancy (266, 270 and 286 pmol/l in the 1st, 2nd and 3rd trimesters respectively) despite the participants not taking B12 or multivitamin supplements (Marzan et al. 1971). The 2 studies which reported median B12 values showed a fall of 13pmol/l and 64pmol/l between the 2nd and 3rd trimesters (Milman et al. 2006; C. S. Yajnik et al. 2008).

*Standardised Score*

A total of 32 of the 57 studies reported mean B12 values in addition to the insufficiency rates. Ten of them were longitudinal studies, providing results from more than one trimester, thus a total of 43 results were available. Additionally, 12

studies reported the median values which were used to estimate the mean. Two of these were longitudinal, yielding a total of 14 results. Combining the above two, we obtained 57 standardised scores (ratio of mean ÷ cut-off level). Details of these studies with their standardised scores and corresponding B12 insufficiency rates are shown in Table 4.2.

**Table 4.2** Calculated Standardised scores and corresponding B12 insufficiency rates

| Study reference | Trimester (n) | Mean B12 level (pmol/l)[1] | B12 cut-off level (pmol/l) | % B12 Insufficiency | Standardised score |
|---|---|---|---|---|---|
| Katre et al. 2010 | 2 (163) | 119[1] | 150 | 73 | 0.79 |
| Koc et al. 2006 | 3 (180) | 96 | 118 | 72 | 0.81 |
| Balci et al. 2014 | 3 (72) | 120 | 148 | 70.8 | 0.81 |
| Yajnik et al. 2008 (b) | 3 (594) | 122[1] | 150 | 71 | 0.81 |
| Ackurt et al. 1995 (b) | 3 (87) | 95 | 111 | 80.9 | 0.85 |
| Yajnik et al. 2008 (a) | 2 (638) | 135[1] | 150 | 60 | 0.9 |
| Hall et al. 2007 | 3 (95) | 180 | 185 | 58.9 | 0.97 |
| Guerra-Shinoara et al. 2004 | 3 (117) | 130 | 132 | 52.9 | 0.98 |
| Shields et al. 2011 (b) | 3 (77) | 153 | 156 | 60 | 0.98 |
| Samuel et al. 2013 | 1 (352) | 149[1] | 150 | 51.1 | 1 |
| Halicioglu et al. 2012 | 3 (208) | 120[1] | 118 | 47.6 | 1.02 |
| Milman et al. 2006 (b) | 3 (256) | 161[1] | 150 | 42.6 | 1.07 |
| Krishnaveni et al. 2009 | 3 (774) | 162[1] | 150 | 43 | 1.08 |
| Roberts et al. 1973 (b) | 3 (119) | 134 | 118 | 48 | 1.14 |
| Yusufji et al. 1973 | 3 (998) | 117 | 103 | 52 | 1.14 |
| Heppe et al. 2013 | 1 (2173) | 175[1] | 150 | 26 | 1.17 |
| Frery et al. 1992 | 3 (188) | 175 | 148 | 27.6 | 1.18 |
| Hussein et al. 2009 | 3 (84) | 185 | 150 | 46.4 | 1.24 |
| Ackurt et al. 1995 (a) | 2 (129) | 141 | 111 | 48.8 | 1.27 |
| Kosus et al. 2012 | 1 (228) | 200[1] | 156 | 12.5 | 1.28 |
| Shields et al. 2011 (a) | 1 (113) | 215 | 156 | 16 | 1.38 |
| Shamim et al. 2013 | 1 (285) | 206 | 150 | 19.6 | 1.38 |
| House et al. 2000 | 2 (1424) | 180[1] | 130 | 25.3 | 1.38 |
| Roberts et al. 1973 (a) | 1 (320) | 165 | 118 | 35 | 1.4 |
| Osifo et al. 1976 | 3 (50) | 208 | 148 | 40 | 1.41 |
| Vanderjagat et al. 2009 | 3 (98) | 208[1] | 148 | 12.2 | 1.41 |
| Baker et al. 1975 | 3 (174) | 85 | 59 | 23 | 1.44 |
| Abdelrahim et al. 2009 | 3 (55) | 159 | 111 | 1.1 | 1.44 |
| Lowenstein et al. 1960 (b) | 3 (252) | 221 | 148 | 19 | 1.49 |
| Murphy et al. 2007 (c) | 3 (84) | 224 | 150 | 0 | 1.49 |
| Milman et al. 2006 (a) | 2 (406) | 225[1] | 150 | 15 | 1.5 |

| | | | | | |
|---|---|---|---|---|---|
| Wu et al. 2013 (b) | 3 (220) | 224 | 148 | 23 | 1.51 |
| Giugliani et al. 1984 | 3 (165) | 251 | 165 | 21.6 | 1.52 |
| Murphy et al. 2007 (b) | 2 (90) | 230 | 150 | 0 | 1.53 |
| Jiang et al. 2005 | 1 (1158) | 237 | 150 | 28.3 | 1.58 |
| Lowenstein et al. 1960 (a) | 2 (59) | 235 | 148 | 15.2 | 1.59 |
| Zachau-Christiansen et al. 1962 | 3 (365) | 177 | 111 | 17 | 1.59 |
| Jacquemyn et al. 2014 | 3 (78) | 244 | 150 | 13 | 1.63 |
| Bjorke-Monsen et al. 2001 | 3 (169) | 245[1] | 150 | 15 | 1.63 |
| Whiteside et al. 1968 (b) | 2 (50) | 127 | 74 | 25 | 1.72 |
| Murphy et al. 2007 (a) | 1 (88) | 267 | 150 | 0 | 1.78 |
| Colman et al. 1975 | 3 (106) | 524 | 295 | 0.9 | 1.78 |
| Takimoto et al. 2007 (b) | 3 (82) | 265 | 148 | 16 | 1.79 |
| Gibson et al. 2008 | 3 (83) | 268[1] | 150 | 23 | 1.79 |
| Cole et al. 1974 | 3 (130) | 272 | 148 | 12.3 | 1.84 |
| Pagan et al. 2002 (b) | 3 (285) | 285 | 148 | 2.1 | 1.93 |
| Wu et al. 2013 (a) | 2 (264) | 287 | 148 | 10 | 1.94 |
| Ray et al. 2008 | 1 (2490) | 249 | 125 | 8.5 | 1.99 |
| Takimoto et al. 2007 (a) | 2 (77) | 301 | 148 | 8 | 2.03 |
| Ho et al. 1987 | 3 (221) | 229 | 110 | 3.6 | 2.08 |
| Knight et al. 1991 (b) | 3 (75) | 318 | 148 | 11.2 | 2.15 |
| Pagan et al. 2002 (a) | 2 (285) | 357 | 148 | 0.35 | 2.41 |
| Jacob et al. 1976 | 2 (182) | 303 | 111 | 4.5 | 2.73 |
| Whiteside et al. 1968 (a) | 1 (56) | 217 | 74 | 5 | 2.93 |
| Black et al. 1994 | 3 (85) | 228 | 74 | 15 | 3.08 |
| Marzan et al. 1971 (a) | 2 (100) | 270 | 59 | 1.5 | 4.58 |
| Marzan et al. 1971 (b) | 3 (57) | 286 | 59 | 0 | 4.85 |

Table showing the relationship between the calculated Standardised Score (mean B12 deficiency ÷ cut-off level) and corresponding B12 insufficiency rate (n=57 pairs of results). The studies are presented according to the standardised score for ease of comparison. The lower case letters in parenthesis in the first column indicate the order of appearance in the table for studies which have reported results from more than one trimester

[1] Mean estimated from median (all such values) (Hozo et al. 2005)

Linear regression was done to test the degree to which standardised score was as a predictor of percentage B12 insufficiency reported in each study, in a model that included trimester, geographical region and assay type. Two outlying results from a single study which gave very high standardised scores of >4.5 (due to a very low B12 cut-off threshold of 59 pmol/l) were excluded (Marzan et al. 1971). The model explained 72% of variance in the percentage insufficiency and the standardised score was the only significant predictor of the former (adjusted **B**-coefficient (95% CI) = -136.5 (-159.7, -113.3) p<0.001) (Figure 4.5). This confirms that, after internal correction for the assay type and cut-off level, it was the same group of studies with the lower standardised scores (from specific geographical regions, namely Indian sub-continent and Eastern Mediterranean) that also found higher prevalence rates of B12 insufficiency, giving more weight to the results seen in the Forest plots (Figures 4.2 – 4.4).

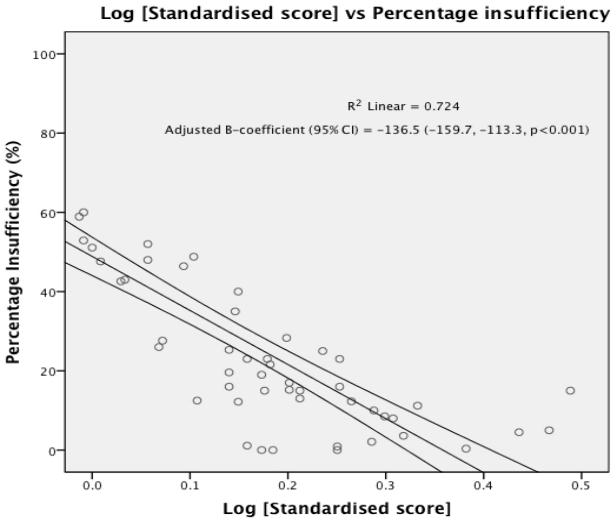


**Figure 4.5** Correlation between log-transformed standardised score and corresponding B12 insufficiency rate. Data are from 55 pairs of results (2 outlying results from a single study with standardised scores of >4.5 due to extremely low B12 thresholds removed). Stepwise linear regression performed with the model adjusted for trimester of sampling, geographical region and assay type.

*Study quality assessment*

Studies were of differing quality and the detailed quality assessments are shown in Appendix 1.2. 23 of the 57 of the studies were of good quality with minimal bias in reporting, a further 28 of moderate quality and six of poor quality. When the breakdown of assessment criteria was looked at, most studies had a clearly defined

question and outcomes but performed poorly in reporting the proportion of eligible participants consenting, reliability and validity of biochemical assays used and in accounting for confounders in analysis.

### 4.3.2. B12 insufficiency and BW sub-review

*Study characteristics*

1900 citations were obtained from the electronic database searches and reviewing references of articles (Figure 4.1B). 947 records were shortlisted for title and abstract screening, of which 148 full-text reviews were done. A total of 23 studies met all the inclusion criteria after full text review and were included in the final analysis (Abbas et al. 1994; Abraham et al. 2013; H. Baker et al. 1977; Dwarkanath et al. 2013; Faintuch et al. 2009; Frery et al. 1992; Furness et al. 2013; Gomes et al. 2010; Guerra-Shinohara et al. 2004; Hay et al. 2010; Hogeveen et al. 2010; Krishnaveni et al. 2014; Lindblad et al. 2005; Ramoteme L Mamabolo et al. 2006; McGarry and Andrews 1972; Muthayya et al. 2006b; Navarro et al. 1984; Pagán et al. 2002; Relton et al. 2005; Sukla et al. 2013; Takimoto et al. 2007; Ubeda et al. 2011; C. S. Yajnik et al. 2005). Tables 4.3-4.5 provides the characteristics of these studies and their results. The studies reported the association between maternal or cord vitamin B12 levels and low birthweight in three different ways: a) logistic regression to analyse the odds ratios (OR) of having an adverse BW outcome (e.g. LBW, SGA or IUGR) with low B12 levels (n=5, Table 4.3); b) comparison of mean/median B12 between adverse BW cases and normal BW controls (n=15, Table 4.4) and c) linear regression to study the association between B12 and birthweight as continuous variables (n=10, Table 4.5). Six studies reported their results in a combination of the above three methods and were included as appropriate (Abraham et al. 2013; Furness et al. 2013; Hogeveen et al. 2010; Krishnaveni et al. 2014; Muthayya et al. 2006b; Sukla et al. 2013).

**Table 4.3** Odds of SGA or LBW in low B12 levels

| Author, date | Country | Trimester[1] | B12 threshold | Birth outcome threshold | Cases (n) | Controls (n) | OR (95% CI) | Adjustments |
|---|---|---|---|---|---|---|---|---|
| Muthayya et al. 2006 (a) | India | 1 | Tertile 1 vs. tertile 3 (median 116 vs. 224pmol/l) | IUGR: <10$^{th}$ centile for GA | 45 | 45 | 5.98 (1.72, 20.74) | Maternal age, education, parity, weight |
| Dwarkanath et al. 2013 (a) | India | 1 | Tertile 1 vs. tertile 3 (median 118 vs. 284pmol/l) | SGA: <10$^{th}$ centile for GA | 107 | 103 | 1.43 (1.02, 2.17) | Age, education, parity, weight, energy intake |
| Muthayya et al. 2006 (b) | India | 2 | Tertile 1 vs. tertile 3 (median 113 vs. 210pmol/l) | IUGR: <10$^{th}$ centile for GA | 50 | 54 | 9.28 (2.90, 29.68) | Maternal age, education, parity, weight |
| Furness et al. 2013 | Australia | 2 | N/R | EFW <10$^{th}$ centile and serial tapering down of abdominal circumference | 21 | 63 | 1.001 (0.996, 1.006) | Folate, Hcy, age, smoking, BMI, DNA damage markers (e.g. micronuclei, nucleoplasmic bridges) |
| Dwarkanath et al. 2013 (b) | India | 2 | Tertile 1 vs. tertile 3 (median 108 vs. 245pmol/l) | SGA: <10$^{th}$ centile for GA | 96 | 96 | 1.45 (0.92, 2.27) | Age, education, parity, weight, energy intake |
| Muthayya et al. 2006 (c) | India | 3 | Tertile 1 vs. tertile 3 (median 111 vs. 182pmol/l) | IUGR: <10$^{th}$ centile for GA | 49 | 53 | 2.81 (1.01. 7.87) | Maternal age, education, parity, weight |
| Hogeveen et al. 2010 | Netherlands | 3 | <134pmol/l (Q1) | LBW: <3075g (Q1) | 92 | 274 | 0.70 (0.44, 1.11) | GA, smoking, sex |
| Sukla et al. 2013 | India | Cord | N/R | LBW: <2500g | 138 | 196 | 2.41 (1.34, 4.5) | N/R |

Table showing the study characteristics and results from studies that describe an association between maternal or cord B12 and birth outcomes by odds ratios (n=5 studies, 8 results). The studies are presented according to the trimesters of pregnancy. The lower case letters in parenthesis in the first column indicate the order of appearance in the table for studies which have reported results from more than one trimester

[1] Refers to trimester of maternal B12 unless specified as Cord; GA – gestational age; IUGR – intrauterine growth retardation; LBW – low birth weight; N/R – not reported; Q1 – quartile 1; SGA – small for gestational age

**Table 4.4** Differences in mean/median B12 levels in normal and adverse birthweight

| Author, date | Country | Trimester[1] | Adverse BW outcome threshold | Adverse BW outcome | | Normal birth outcome | |
|---|---|---|---|---|---|---|---|
| | | | | n | Mean (SD) *pmol/l* | n | Mean (SD) *pmol/l* |
| Muthayya et al. 2006 (a) | India | 1 | LBW: <2500g<br>NBW: >3000g | 16 | 156 (65) | 39 | 173 (58) |
| Ubeda et al. 2011 (a) | Spain | 1 | IUGR: BW <10$^{th}$ centile for GA | 7 | 227.5 (132.4) | 48 | 260.4 (124.8) |
| McGarry et al. 1972 | UK | 2 | LBW: <2500g<br>NBW: >2950g | 14 | 120 (33) | 331 | 136 (53) |
| Muthayya et al. 2006 (b) | India | 2 | LBW: <2500g<br>NBW: >3000g | 19 | 139 (33) | 44 | 163 (46) |
| Ubeda et al. 2011 (b) | Spain | 2 | IUGR: BW <10$^{th}$ centile for GA | 7 | 194.6 (75.9) | 48 | 209.5 (105.8) |
| Furness et al. 2013 | Australia | 2 | IUGR: EFW <10$^{th}$ centile and serial tapering down of abdominal circumference | 21 | 205 (87.9) | 63 | 243 (135) |
| Krishnaveni et al. 2014 | India | 2 | LBW: <2500g | 126 | 191 (93) | 528 | 186 (102) |
| Baker et al. 1977 (a) | USA | 3 | LBW: <2500g | 50 | 95 (13) | 50 | 78 (13) |
| Navarro et al. 1984 (a) | France | 3 | LBW: <2500g | 31 | 295 (90) | 26 | 311 (58) |
| Abbas et al. 1994 (a) | UK | 3 | IUGR: abdominal circumference and EFW <5$^{th}$ centile | 20 | 0.1 (0.21)[2] | 20 | N/A[2] |
| Lindblad et al. 2005 (a) | Pakistan | 3 | IUGR: EFW ≤ 11% | 46 | 96 (41)[3] | 82 | 108 (48)[3] |
| Yajnik et al. 2005 | India | 3 | SGA: <10$^{th}$ centile for sex and GA | 30 | 106 (87, 128)[4] | 50 | 124 (100, 150)[4] |
| Mamabolo et al. 2006 | South Africa | 3 | Tertile 1 vs. tertile 3 | 66 | 176 (74) | 75 | 175 (78) |
| Muthayya et al. 2006 (c) | India | 3 | LBW: <2500g<br>NBW: >3000g | 19 | 137 (38) | 42 | 156 (45) |
| Ubeda et al. 2011 (c) | Spain | 3 | IUGR: BW <10$^{th}$ centile for GA | 7 | 139.9 (44.0) | 48 | 161.0 (95.6) |
| Abraham et al. 2013 | India | 3 | LBW: <2500g | 58 | 207 (94) | 58 | 203 (87) |
| Baker et al. 1977 (b) | USA | Cord | LBW: <2500g | 50 | 281 (43) | 50 | 439 (35) |
| Navarro et al. 1984 (b) | France | Cord | LBW: <2500g | 32 | 223 (61) | 26 | 255 (56) |
| Abbas et al. 1994 (b) | UK | Cord | IUGR: abdominal circumference and EBW <5$^{th}$ centile | 20 | 0.9 (0.28)[2] | 20 | N/A[2] |
| Lindblad et al. 2005 (b) | Pakistan | Cord | IUGR: EFW ≤ 11% | 46 | 190 (142)[3] | 82 | 171 (81)[3] |
| Muthayya et al. 2006 (d) | India | Cord | LBW: <2500g<br>NBW: >3000g | 20 | 195 (63) | 47 | 236 (94) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Gomes et al. 2010 | Sri Lanka | Cord (preterm infants) | SGA: BW <10$^{th}$ centile for GA and sex | 96 | 394 (169.3) | 113 | 409 (224.5) |
| Hay et al. 2010 (a) | Norway | Cord (nulliparous) | Quartile 1 vs. quartile 4 | 180 | 363 (341, 420)[5] | 180 | 242 (221, 311)[5] |
| Hay et al. 2010 (b) | Norway | Cord (multiparous) | Quartile 1 vs. quartile 4 | 180 | 365 (301, 423)[5] | 180 | 258 (224, 297)[5] |
| Sukla et al. 2013 | India | Cord | LBW: <2500g | 138 | 142.4 (60.5) | 196 | 157.9 (53.9) |

Table showing the characteristics and results from studies that describe mean maternal or cord B12 levels in adverse and normal birth outcome groups (n=15 studies, 25 results). The studies are presented according to the trimesters of pregnancy. The lower case letters in parenthesis in the first column indicate the order of appearance in the table for studies which have reported results from more than one trimester

[1] Refers to trimester of maternal B12 unless specified as Cord

[2] Mean difference (SEM) in SD between cases and controls

[3] Mean (SD) estimated from median (range) (Hozo et al. 2005)

[4] Geometric mean (25$^{th}$, 75$^{th}$ centile)

[5] Geometric mean (95% CI)

BW - birth weight; EFW – estimated fetal weight; GA – gestational age; IUGR – intrauterine growth retardation; LBW – low birth weight; N/A – not applicable; SGA – small for gestational age

**Table 4.5** Effect of B12 levels across the spectrum on birthweight

| Author, date | Country | Trimester[1] | n | Unit B12 | Effect size (95% CI) | P value | Adjustments |
|---|---|---|---|---|---|---|---|
| Relton et al. 2005 (a) | UK | 1 | 500 | 1 unit log B12 (pg/ml) | BW z-score<br>**β** = 0.03 (-0.05, 0.12) | 0.41 | None |
| Takimoto et al. 2007 (a) | Japan | 1 | 51 | 1 pmol/l | B=-1.05 | 0.08 | Age, parity, BMI |
| Pagan et al. 2002 (a) | USA | 2 | 285 | 1 pmol/l | B=-0.2 | 0.52 | GA, race, BMI, smoking, gender |
| Takimoto et al. 2007 (b) | Japan | 2 | 77 | 1 pmol/l | B=-5.35 | 0.38 | Age, parity, BMI |
| Faintuch et al. 2009 | Brazil | 2 (Post-bariatric surgery) | 13 | 1 pg/ml | r= -0.846 | <0.001 | None |
| Frery et al. 1992 (a) | France | 3 (All) | 188 | 1 unit log B12 | r=-0.05 | NS | Parity, ethnicity |
| | | 3 (Smokers) | 25 | | B=-507 | 0.03 | |
| Pagan et al. 2002 (b) | USA | 3 | 285 | 1 pmol/l | B=-0.2 | 0.53 | GA, race, BMI, smoking, gender |
| Guerra-Shinoara et al. 2004 (a) | Brazil | 3 | 117 | 1 pmol/l | r= -0.05 | 0.52 | None |
| Takimoto et al. 2007 (c) | Japan | 3 | 82 | 1 pmol/l | B=0.776 | 0.44 | Age, parity, BMI |
| Hogeveen et al. 2010 | Netherlands | 3 | 366 | 1 SD (69pmoll/l) | **B**= -37 (-100, 29) | NS | Age, GA, parity, smoking, sex, folate supplement |
| Abraham et al. 2013 | India | 3 | 116 | 1 pmol/l | **β**=0.22 | 0.65 | Diet, socio-economic status |
| Krishnaveni et al. 2014 | India | 3 | 654 | 1 SDS unit log B12 | BW SDS<br>**β**= 1: 0.02 (-0.05,0.10)<br>2: 0.07 (-0.003,0.15) | NS | 1: Sex, gestational age<br>2: 1 + BMI, GDM, SES, parity, religion |
| Frery et al. 1992 (b) | France | Cord (All) | 154 | 1 unit log B12 | r= -0.16 | <0.04 | Parity, ethnicity |
| | | Cord (Smokers) | 22 | | B= -414 | 0.06 | |
| Guerra-Shinoara et al. 2004 (b) | Brazil | Cord | 117 | 1 pmol/l | r= -0.02 | 0.80 | None |
| Relton et al. 2005 (b) | UK | Cord | 522 | 1 unit log B12 (pg/ml) | BW z-score<br>**β**= -0.09 (-0.17, -0.01) | 0.02 | None |
| Muthayya et al. 2006 | India | Cord (37-39/40) | 76 | 1 pg/ml | r= 0.28 | 0.01 | None |
| | India | Cord (>40/40) | 36 | 1 pg/ml | r= -0.13 | 0.45 | None |

Table showing the characteristics and results from studies that describe an association between maternal or cord B12 and birth outcomes by correlation/regression analysis (n=10 studies, 19 results). The studies are presented according to the trimesters of pregnancy. The lower case letters in parenthesis in the first column the order of appearance in the table for studies which have reported results from more than one trimester

[1] Refers to trimester of maternal B12 unless specified as Cord

BMI – body mass index; BW - birthweight; GA – gestational age; GDM – gestational diabetes mellitus; N/R – not reported; SDS – standard deviation score; SES – socio-economic status

*Odds of SGA or LBW in low B12 levels*

Five studies across the trimesters (Dwarkanath et al. 2013; Furness et al. 2013; Hogeveen et al. 2010; Muthayya et al. 2006b) and cord blood (Sukla et al. 2013) (eight results) reported the odds ratio (ORs) of SGA or LBW in lower B12 levels compared to higher levels (Table 4.3). Meta-analysis (n=1482; 598 cases, 884 controls) of all of these studies showed that the odds of having a SGA/LBW baby was 1.70 (95% CI 1.16, 2.50) with lower B12 levels (Figure 4.6).

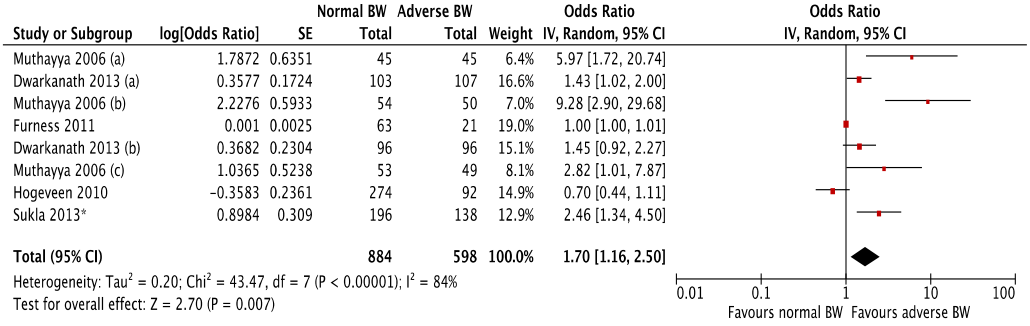


**Figure 4.6** Meta-analysis of OR's of adverse birth weight (BW) in low maternal (or cord) B12 cases and controls (n=8 results). The letters in parantheses after the Study ID refer to results from different trimesters within each study. The black diamond represents the pooled OR while the squares in each study denote the OR for that study (bars are 95% CI). Pooled analysis and heterogenity analysis was done on log-transformed ORs using a random effects model. The pooled OR (95% CI) was 1.70 (1.16, 2.50). * B12 measured in cord blood

There were too few results from different countries and trimesters to provide sub-group analysis. Therefore, we only did the following three analyses: 1) removal of the study reporting only cord blood B12 levels (Sukla et al. 2013) (because B12 is actively transported across the placenta (Obeid et al. 2005) and is expected to higher than that of maternal B12); 2) combining the five results from India (from three studies) (Dwarkanath et al. 2013; Muthayya et al. 2006b; Sukla et al. 2013) due to the high prevalence of both B12 insufficiency and SGA/LBW babies (United Nations Children's Fund and World Health Organization 2004); and 3) removal of the *Muthayya et al* study as it reported large effect sizes and contributed 21.5% to the pooled analysis (Muthayya et al. 2006b) . The ORs (95% CI) were 1.59 (1.07, 2.36), 2.42 (1.50. 3.92) and 1.23 (0.90, 1.67), respectively in these sub-group analyses. There were only two other results (from Australia and Holland), which did not show any association between B12 and BW (Furness et al. 2013; Hogeveen et al. 2010).

*Differences in mean/median B12 levels and adverse birthweight*

Fifteen studies reported either mean or median B12 values between normal and LBW/SGA groups. There were eight cross-sectional and seven longitudinal studies, yielding a total of 25 results (Table 4.4). Two sub-group meta-analysis by maternal levels (n=1969; 487 cases and 1482 controls; 14 results) (Figure 4.7A) and cord blood levels (n=896; 382 cases and 514 controls; six results) (Figure 4.7B) were conducted. Although the pooled estimates showed lower maternal B12 levels in the adverse birthweight group (particularly in the second trimester), these were not statistically significant (Figure 4.7A). Similarly, the pooled meta-analysis of cord blood B12 levels were not different between the birthweight groups (Figure 4.7B).

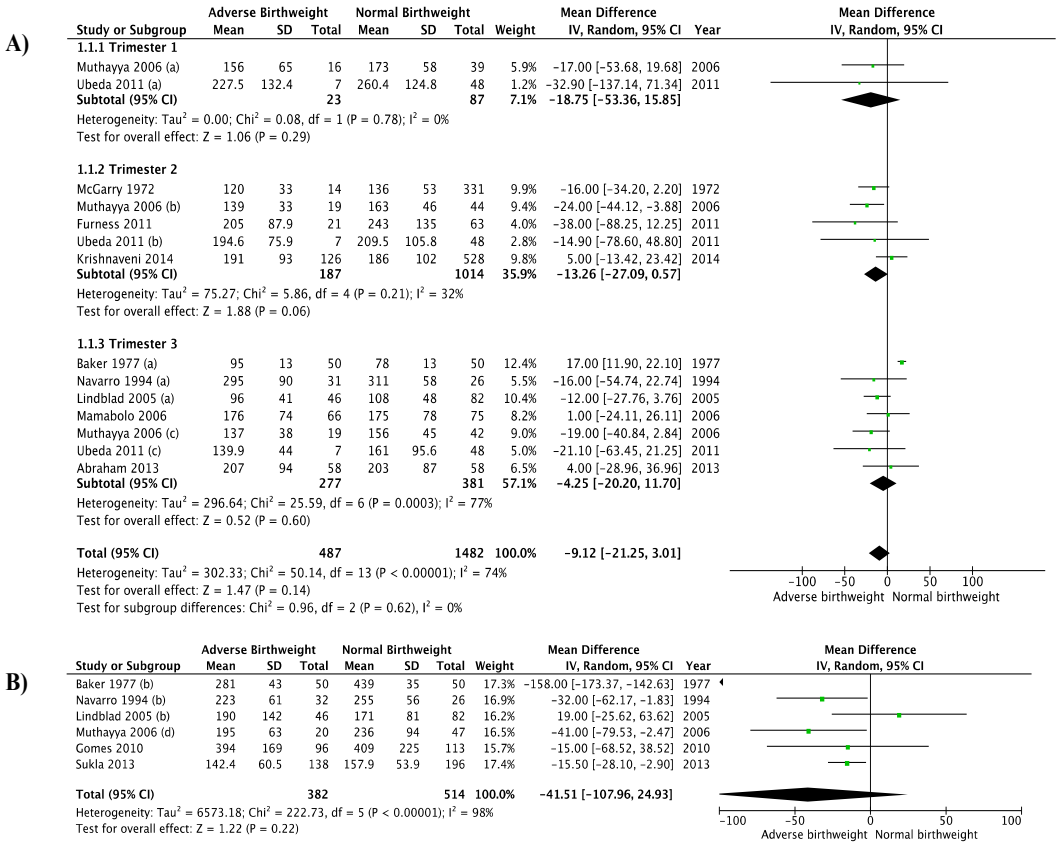


**Figure 4.7** Meta-analysis of difference in mean B12 between adverse and normal birth weight babies. **A)** B12 measured in maternal blood (divided in subgroups according to trimesters of pregnancy) (n=14 results) and **B)** B12 measured in cord blood (n=6 results).
The black diamonds represent the pooled difference for each subgroup and overall while the squares in each study denote the mean difference for that study (bars are 95% CI). The $I^2$ values refer to the statistical heterogenity within each subgroup and overall. A random effects model using generic inverse variance showed a pooled mean difference (95% CI) of -9.12 (-21.25, 3.01) in pregnancy and -41.51 (-107.96, 24.93) in cord blood between adverse and normal birth weight babies.

Three other studies (n=880; 430 cases and 450 controls; 5 results), from the 3rd trimester and cord blood showed differences in B12 levels between the normal and adverse BW groups in terms geometric mean (Hay et al. 2010; C. S. Yajnik et al. 2005), or mean difference in SD (Abbas et al. 1994) (Table 4). One showed non-significantly lower geometric mean B12 level in third trimester in SGA (C. S. Yajnik et al. 2005) but none of the other studies showed the positive association expected. These studies were not included in the pooled analysis of Figures 6A and 6B because the effect sizes could not be converted to a mean (SD).

*Effect of B12 levels across the spectrum on birthweight*

Ten studies examined B12 and birthweight as continuous variables (Table 5). Where the studies performed both unadjusted linear correlations and adjusted regression coefficients we reported the latter (as B- or β-coefficients) but if only unadjusted correlations were carried out, we reported the former (as r-coefficients). One showed a significant positive correlation (Muthayya et al. 2006a) and 9 showed no association between B12 and birth weight. Of these 9, 2 showed positive but non-significant (Abraham et al. 2013; Krishnaveni et al. 2014), 5 negative (Faintuch et al. 2009; Frery et al. 1992; Guerra-Shinohara et al. 2004; Hogeveen et al. 2010; Pagán et al. 2002) and 2 varying associations at different time points (Relton et al. 2005; Takimoto et al. 2007).

*Study quality assessment*

Nine of the 23 studies were of good quality with minimal bias in reporting, a further 10 of moderate quality and four of poor quality (Appendix 1.3). Although the studies generally did well in reporting an appropriate study question and describing the source population, more than half them did not report the number of eligible participants who consented, the rates of mothers/neonates lost to follow-up or do a comparison between participants with available and missing data.

### 4.4. Discussion

*Prevalence of B12 insufficiency*

One of the striking findings of our systematic review was the high rates of B12 insufficiency among certain populations such as those from India, Nepal, Turkey, Greece and parts of South America. The high rates in the studies from the Indian sub-continent can be explained by a predominant vegetarian diet (Krishnaveni et al. 2009). In 3 of the series from this region, only 7 -15% consumed animal products more frequently than every alternate day (Katre et al. 2010; C. S. Yajnik et al. 2008; Yusufji et al. 1973). Sub-group analysis from another Indian cohort found lower B12, higher folate and Hcy levels amongst Hindu women compared to Muslim participants, which can be attributed to their increased intake of non-vegetarian foods (Krishnaveni et al. 2009).

However, we also found high rates of B12 insufficiency in four studies from Turkey and one from Greece (Ackurt et al. 1995; Balci et al. 2014; Halicioglu et al. 2012; Koc et al. 2006; Schulpis et al. 2004). The "Mediterranean diet" contains plenty of fruits, vegetables and pulses compared to meat (Willett et al. 1995). It has been shown to confer health benefits such as increased life expectancy and lower cardiac disease in adults and increased placental and offspring birthweight when consumed by pregnant women (Timmermans et al. 2012; Willett et al. 1995). However one Turkish series found that stricter adherence to the Mediterranean diet among the sub-group of pregnant women exacerbated B12 deficiency (mean B12 in women consuming animal products weekly or less was 123pmol/l vs. 178 pmol/l if consumed every 2-3 days) (Balci et al. 2014). In addition, obesity rates are high in these regions, which are shown to be associated with low B12 (Adaikalakoteswari et al. 2015b; Delibasi et al. 2007; B. A. Knight et al. 2015; Krishnaveni et al. 2009; Pinhas-Hamiel et al. 2006). The link between obesity, gestational diabetes and low B12 levels has not been fully explored but since the former two conditions are independently associated with fetal macrosomia, they may partly compensate for or mask the associations between B12 and LBW (Gaudet et al. 2014; He et al. 2015).

Additionally, rates of metabolic syndrome in non-pregnant adults from Turkey are in the order of 30 to 45%, particularly in women (Gundogan et al. 2013; Kozan et al. 2007). It is possible that one of the reasons for this could be due to repercussions of B12 insufficiency during child-bearing years as these women may have preference for carbohydrate-rich foods over animal products (thereby exacerbating micronutrient insufficiency levels), or to the elevation of homocysteine (Yaman et al. 2009). None of the studies from this region reported the early pregnancy BMI of their participants, but 2 series reported the mean 1$^{st}$ and 3$^{rd}$ trimester weights of their participants, which was 66kg (Kosus et al. 2012) and 77kg (Schulpis et al. 2004) respectively, considerably higher than the 47 - 49 kg women from the Indian cohorts (Katre et al. 2010; Rao et al. 2001; Samuel et al. 2013). This suggests that the aetiology of B12 insufficiency may be different in these 2 groups of women, which needs to be explored further.

Plausible reasons for the observed fall in B12 during pregnancy are hemodilution, active transport to the fetus and changes in binding proteins (Bruinse and van den Berg 1995; Koebnick et al. 2002). Holotranscobalamin (holoTC), the functional form of B12, is positively correlated with total cobalamin and the two biomarkers negatively correlate with methylmalonic acid (a marker of tissue level B12 insufficiency) during pregnancy (Murphy et al. 2007). While serum cobalamin decreases across the trimesters, holoTC has been shown to decrease in some studies and remain unchanged in others (Greibe et al. 2011; Murphy et al. 2007). Therefore, the fall in cobalamin may be due to a reduction in the fraction bound to haptocorrin (holo-haptocorrin (holoHC)) (Greibe et al. 2011). The tissue-level effects and clinical implications of a fall in holoHC during pregnancy are unknown and warrant further studies. There is an argument for the more pervasive use of holoTC measuring assays during pregnancy, which has similar sensitivity and specificity as total cobalamin measurement and may be superior in identifying early and "true" cobalamin deficiency (Hvas and Nexo 2005; Nexo et al. 2002).

*B12 insufficiency and birth weight (BW)*

Our review showed that the odds of LBW is 1.7 when B12 insufficiency was present in maternal or cord blood (Figure 4.6). However, our results cannot confirm the association between low B12 and LBW across the world as any positive observations may be isolated to Indian populations. Whilst all the studies adjusted for most of the known confounding factors, the overall effect was driven by one study in India (Muthayya et al. 2006b). It is important to note that only 27% of the women recruited into the study had B12 levels measured although the authors reported that they did not differ from the study population in their baseline characteristics. The three results from this study contributed a total of 21.5% to the pooled results (Figure 4.6) and when they were removed from the analysis the OR decreased from 1.70 to 1.23 (95% CI 0.90, 1.67). Given the potential importance of the problem, further studies are needed to replicate or refute the magnitude of association found in this study.

With regards to the two studies from Australia and Holland with negative results, it is notable that when compared to the Indian studies, the maternal B12 concentration in the Australian study was considerably high (median 239pmol/l) while in the Dutch study a higher threshold was used to define LBW (<3075g). Additionally, the population characteristics differed (e.g. mean BMI of women in the Australian study was 28.5 $kg/m^2$ compared to 22.0 $kg/m^2$ in the Indian studies), which may have influenced their risk of having LBW/SGA babies (Dwarkanath et al. 2013; Furness et al. 2013; Muthayya et al. 2006b).

In the studies reporting mean levels of B12, a non-significant trend of lower B12 levels was observed in women who delivered LBW or SGA babies with a larger effect size found in the 1st and 2nd compared to the 3rd trimester. Since the heterogenity between the studies was high and there were differences in the populations and B12 assays, it was not possible to make meaningful conclusions about a B12 'threshold' which would be associated with lower fetal BW. In the 2 studies which reported results from more than one trimester, the difference in maternal B12 between normal and low BW babies was greater in the 1st or 2nd trimester compared to the 3rd (-33 vs. -21 and -24 vs. -19pmol/l respectively) suggesting that lower B12 earlier in pregnancy may be more detrimental for offspring weight (Muthayya et al. 2006b; Ubeda et al. 2011).

The link between exposure to low B12 conditions during pregnancy, BW and non-communicable diseases in the offspring can be explained by the developmental origins of health and disease (DOHaD) hypothesis, which suggests that the fetus is programmed to adapt to its in-utero environment and if this is altered, can result in disease (C. S. Yajnik and Deshmukh 2012). The epigenetic modifications associated with low B12 levels influence placental development from the early embryonic stage, so it is possible that B12 has an effect on fetal growth and BW through this mechanism (Koukoura et al. 2012; Waterland and Jirtle 2004). B12 also plays an important role in myelination of fetal neurons, which maximally occurs from mid-gestation until two years of age (M. M. Black 2008). Hence the critical window for B12 adequacy continues throughout pregnancy and lactation.

*Impact of maternal B12 insufficiency on neonatal and childhood health*

Aside from BW, B12 insufficiency during pregnancy has been linked to other adverse neonatal outcomes such as preterm delivery and pre-eclampsia, which may further compound the problem of sub-optimal BW and poor neonatal outcomes in these babies (Makedos et al. 2007; Ronnenberg et al. 2002). Additionally, cobalamin deficiency in infants can lead to neurological disturbances such as developmental regression and poor intellectual performance in the long-term (Graham et al. 1992).

Although low birthweight is itself a marker for increased risk of cardiometabolic disease in adulthood, authors have also tried to prove an association between exposure to low B12 levels in utero and associated adverse outcomes in childhood or adolescence. The follow-up of the Pune study found that low maternal B12 at 18 weeks gestation (more specifically the combination of low B12 and high folate) was related to insulin resistance in 6 year old offspring and a similar positive association between 1$^{st}$ trimester B12 deficiency and HOMA-IR in children aged 6-8 years was found in a study from Nepal (Stewart et al. 2011; C. S. Yajnik et al. 2008). However, another cohort study from India was not able to replicate these findings when the children were examined up to 13.5 years (although maternal Hcy was positively associated with offspring glucose) (Krishnaveni et al. 2014). What is notable is that in the former 2 studies, the B12 was measured in before 20 weeks gestation compared to 30 weeks gestation in the latter study so it is plausible that fetal exposure to sub-optimal B12 early in pregnancy may have the most deleterious effect on future metabolic health, especially if coupled with high maternal folate towards to the end of pregnancy (Krishnaveni et al. 2014; C. S. Yajnik et al. 2008). In keeping with this theory are the results of our meta-analysis, which found that the strongest association between low B12 levels and LBW was found in the 2$^{nd}$ trimester.

*Changes in biomarkers related to B12 during pregnancy*

Previous studies have shown an independent inverse relationship between maternal homocysteine levels and LBW (OR 1.25, B-coefficient=-31g per 1 SD increase in Hcy) and hyperhomocysteinaemia has been causally linked to LBW (Hogeveen et al. 2012; C.

S. Yajnik et al. 2014). In our Prevalence sub-review, 14 of the 57 studies reported Hcy levels (Barbosa et al. 2008; Bjorke Monsen et al. 2001; Gibson et al. 2008; Guerra-Shinohara et al. 2004; Hall et al. 2007; Katre et al. 2010; Milman et al. 2006; Pagán et al. 2002; Park et al. 2004; Samuel et al. 2013; Takimoto et al. 2007; D. Vanderjagt et al. 2009; Wu et al. 2013; C. S. Yajnik et al. 2008). While a detailed discussion of the associations between B12 and Hcy is beyond the scope of this review, it is notable that among the studies with high B12 insufficiency rates (>40%), the average Hcy was >6 umol/l and in one study, up to 40% of the women had Hcy >10 umol/l (Hall et al. 2007; Park et al. 2004; Samuel et al. 2013; C. S. Yajnik et al. 2008). It is therefore possible that the association of low B12 status and LBW may be, at least in part, mediated through hyperhomocysteinaemia during pregnancy.

Since serum homocysteine (Hcy) is elevated in both B12 and folate deficiency, it has a lower specificity in the presence of B12 deficiency while serum methylmalonic acid (MMA) is more specific to detect B12 deficiency specifically (D. Vanderjagt et al. 2009). 10 studies in our review reported MMA levels (Barbosa et al. 2008; Bjorke Monsen et al. 2001; Gibson et al. 2008; Guerra-Shinohara et al. 2004; Hall et al. 2007; Milman et al. 2006; Samuel et al. 2013; D. Vanderjagt et al. 2009; Wu et al. 2013; C. S. Yajnik et al. 2008). One study that did detailed analysis on the biochemical consequences of cobalamin insufficiency found that maternal B12 was the strongest predictor of low B12 (OR 7.5, $p<0.001$), high Hcy (OR 11.8, $p<0.001$) and high MMA (OR 5.2, $p<0.001$) in newborns (Bjorke Monsen et al. 2001). A robust statistical method to determine at which point on the range of B12 concentrations tissue level deficiency would develops is by doing receptor operator curve analysis. A study which did this in pregnancy (also included in our review) showed that a combination of elevated Hcy and SAM:SAH ratio had the highest sensitivity and specificity to detect B12 deficiency and the corresponding cut-off level of B12 was 132 pmol/l (Guerra-Shinohara et al. 2004).

In the absence of folate deficiency, B12 deficiency is the strongest driver of high Hcy levels (Selhub et al. 2007b). In 12 studies with high rates of B12 insufficiency, folate deficiency was less than 10% (Balci et al. 2014; A. K. Black et al. 1994; Gibson et al.

2008; Giugliani et al. 1984; Hall et al. 2007; House et al. 2000; Katre et al. 2010; Koc et al. 2006; Krishnaveni et al. 2009; Park et al. 2004; Schulpis et al. 2004; C. S. Yajnik et al. 2008). This observation was possibly due to adequate dietary intake and antenatal consumption of folic acid (Balci et al. 2014; Hall et al. 2007; Katre et al. 2010; Park et al. 2004; Schulpis et al. 2004; C. S. Yajnik et al. 2008). This imbalance between B12 and folate is associated with lower neonatal BW and anthropometry as well as insulin resistance in offspring (Gadgil et al. 2014; C. S. Yajnik et al. 2008). Therefore it is essential to address maternal B12 status in addition to folate during pregnancy.

A meta-analysis of multiple micronutrient supplementation trials (typically containing one recommended daily allowance of B12) done in 12 low-income countries showed that supplementation was associated with modest increase in BW (effect size +22g. p=0.002) and reduced LBW and SGA (Fall et al. 2009). While this may not be due to optimising levels of B12 per se, it suggests that micronutrients in general are likely to contribute to increasing BW. Folic acid supplementation has been associated with higher BW, supporting the above explanation (Christian et al. 2003; Hodgetts et al. 2015).

The strengths of our study are that this is the first review, to our knowledge, to consider the B12 status of pregnant women on a global level and link this to BW. We were able to demonstrate patterns in B12 insufficiency rates and associations with BW in populations who broadly share dietary habits and inherent risk although finer differences may exist. One key limitation of our report is the vast heterogenity between the studies in terms of B12 measurement assays and cut-off levels. This was partly addressed by devising the 'standardised score' which allowed comparisons to be made between the studies, after controlling for geographical region, trimester and assay type. Another limitation is that despite the large number of studies, the numbers were small in sub-groups (such as individual trimesters), highlighting the need for adequately powered longitudinal, cohort studies with LBW or SGA as outcomes.

### 4.5. Conclusion

Our systematic review and meta-analysis in pregnant women showed that rates of vitamin B12 insufficiency are high in certain populations (e.g. the Indian sub-continent and Eastern Mediterranean), including in non-vegetarian ones. The possible association between B12 insufficiency and LBW/SGA warrants further investigation through larger cohort studies and randomised controlled trials. Even if the effect size of maternal B12 on BW is modest, it has potential to influence the health of future generations if a link is proven. The results of further studies will dictate practice with regards to B12 supplementation in pre-conception and pregnancy but until then, it would be sensible at least to measure B12 levels when pregnant women first present to antenatal facilities across the world.

# Chapter 5

# Vitamin B12 status among pregnant women in the UK and its association with obesity and gestational diabetes

## 5.1. Introduction

The burden of maternal obesity (defined as body mass index (BMI) greater than 30 $kg/m^2$) is rapidly increasing, affecting nearly 20% of pregnant women in the UK (Heslehurst et al. 2010). High BMI is associated with adverse pregnancy outcomes including recurrent miscarriages and maternal deaths (Lewis 2007). In parallel, the incidence of gestational diabetes (GDM) has also risen affecting 5-18 % of all pregnancies depending on the diagnostic criteria applied (Buckley et al. 2012; Cundy et al. 2014).

In Chapter 3, it was described how the mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA requires B12 as a coenzyme and in its absence, accumulation of the former compound inhibits fatty acid oxidation, thereby promoting lipogenesis (Adaikalakoteswari et al. 2014; Brindle et al. 1985). Therefore it can be postulated that low B12, at a cellular level, may be linked to adipocyte dysfunction and obesity-related complications by modulating lipid metabolism, cellular inflammation (Kumar et al. 2013) and causing hypomethylation of cholesterol biosynthesis pathways (Adaikalakoteswari et al. 2015b).

Low B12 during pregnancy has implications for materno-fetal health including maternal adiposity, maternal and offspring insulin resistance (Krishnaveni et al. 2009; Stewart et al. 2011; C. S. Yajnik et al. 2008) and adverse lipid profile in neonates (Adaikalakoteswari et al. 2015a; Adaikalakoteswari et al. 2015b). The first two observations were replicated in a cohort of women without gestational diabetes mellitus (GDM) from South West England (B. A. Knight et al. 2015) but there are no data available on the role of B12 in GDM in the UK.

Aside from the possible association with low birthweight (LBW) seen in Chapter 4, suboptimal B12 levels in pregnancy has also been shown to be independently associated with adverse lipid profile in neonates (Adaikalakoteswari et al. 2015a) and higher insulin resistance in children (C. S. Yajnik et al. 2008). LBW or small for gestational age (SGA) are outcomes of particular interest as they are well-established surrogate markers for

metabolic disorders such as obesity, type 2 diabetes and metabolic syndrome in later life in many populations (Hales and Barker 2001; Whincup et al. 2008; C.S. Yajnik et al. 1995). At the other end of the spectrum, maternal obesity and insulin resistance are well-known to be associated with higher fetal birthweight (Gaudet et al. 2014; He et al. 2015). Since B12 may be inversely associated with maternal BMI (B. A. Knight et al. 2015; Krishnaveni et al. 2009), it is possible that B12 is an independent mediator or a confounder for high birthweight.

The hypothesis of this chapter is that B12 insufficiency is common among pregnant women in a UK population and it may be associated with GDM and other surrogate materno-fetal factors.

The primary aim of our study is to investigate B12 and folate status of pregnant women in the UK and their relationship with obesity and GDM and secondarily to assess their relationship with fetal birthweight.

## 5.2. Methods

### 5.2.1. Study design and justification

A retrospective case-control study of pregnant women attending the antenatal clinic in a district general hospital in the West Midlands, UK, between 2010 and 2013 was conducted. The justification for selecting this method of study is because there is no data available, in a UK population, about B12 status among pregnant women at high risk of, or diagnosed with GDM and associated effects. Therefore, a pilot study such as this would be informative prior to conducting any prospective studies investigating the same.

### 5.2.2. Participant selection

Using the hospital information database which had routine materno-fetal records of all deliveries during this period, we identified women who had a diagnosis of GDM and those who did not (labelled as no-GDM) and had their B12 levels measured in the 2$^{nd}$ or 3$^{rd}$ trimesters of the pregnancy. The no-GDM group consisted predominantly of women attending the medical obstetrics clinic for varying medical conditions. B12 and folate

levels were measured routinely for screening for anaemia by the medical obstetric lead (VP), in addition to haemoglobin and ferritin. The physician (PS) running the antenatal-diabetes clinic measured these micronutrient levels for similar reasons in their first visit after the diagnosis of GDM. The following women were excluded from our analysis: pre-gestational diabetes (Type 1 and 2), multiple pregnancies and those on vitamin B12 supplements at the time of blood sampling.

### 5.2.3. Data collection and laboratory analysis

Clinical information about the women including medical and pregnancy history, smoking status and BMI at booking was recorded from the database. Fetal outcomes such as birthweight, sex and gestation were obtained for the secondary outcome analysis. Analysis of glucose was done by a hexokinase enzymatic method in the hospital laboratory and serum B12 and folate by an electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyser (Roche Diagnostics UK, Burgess Hill, UK).

### 5.2.4. Definitions of outcomes

A selective screening approach was used to screen high-risk women for GDM according to the NICE guidelines (i.e. BMI >30kg/$m^2$, previous GDM, previous macrosomia, first degree relative with diabetes, ethnic minority race) (National Institute for Health and Care Excellence 2008). This consisted of a 2-hour 75g glucose tolerance test between 26-28 weeks of gestation. The modified WHO 1999 criteria was used to diagnose GDM (fasting glucose ≥6.1mmol/l or 2-hour glucose ≥7.8mmol/l) during the study period. The reference range for serum B12 was 150 - 489 pmol/l and serum folate 7.0 – 42.4 nmol/l respectively. Insufficiency of the two micronutrients were defined as <150pmol/l and <7 nmol/l respectively (B. A. Knight et al. 2015; Krishnaveni et al. 2009). Birthweight centiles and z-scores were calculated using gestational age at delivery and sex-specific reference standards published by the Intergrowth calculator 21$^{st}$ Project (Villar et al. 2014). Macrosomia was defined as birthweight >4000g, large for gestational age (LGA) as > 90$^{th}$ centile for sex and gestational age, LBW as <2500g and small for gestational age (SGA) as <10$^{th}$ centile for sex and gestational age.

### 5.2.5. Statistical analysis

Based on pilot data, the required sample size in each group to demonstrate a 15% difference in mean B12 with 90% power and at 5% significance, was calculated to be 144. Statistical analysis was performed using SPSS version 22.0 (IBM Corp Released 2013). Since BMI, serum B12 and folate were not normally distributed, they were log-transformed for statistical purposes. For comparison of GDM and no-GDM mothers, the Student's t-test was used for continuous variables (e.g. B12, folate and BMI) and the chi-square test for categorical variables. Stepwise multiple linear regression was performed with B12 and folate as the dependent variables with the predictors entered or removed from the model according to the following criteria: Probability-of-F-to-enter $\leq 0.050$, Probability-of-F-to-remove $\geq 0.100$. Logistic regression was done to determine the odds of maternal obesity and GDM according to B12/folate insufficiency status and the risk of macrosomia, LGA, LBW and SGA according to quartiles of B12/folate. The regression models included the following co-variates: age, parity, ethnic origin, smoking, gestation of bloods, BMI, B12 and folate (where appropriate). For macrosomia and LBW, sex and gestational age were additionally added to the models.

### 5.2.6. Ethics approval

Our institution has obtained ethics approval to collect B12 and folate data from pregnant women in an anonymised form (NHS ethics committee reference number 12/LO/0239).

## 5.3. Results

### 5.3.1. Overall study parameters

Out of approximately 8400 deliveries in the hospital between 2010-13 which were screened, 344 women (143 GDM, 201 no-GDM) who met the inclusion criteria and had B12 levels measured in the 3$^{rd}$ trimester of pregnancy were included. The clinical characteristics of the whole cohort and by GDM status are provided in Table 5.1. Of the 201 no-GDM women, 45% had OGTT as per NICE selective screening criteria (National Institute for Health and Care Excellence 2008) and the characteristics of all the no-GDM women are summarised in Appendix 2.1.

**Table 5.1** Maternal characteristics according to GDM status.

| Variables | Total | GDM | No GDM |
|---|---|---|---|
| Number (%) | 344 (100) | 143 (41.6) | 201 (58.4) |
| Age (yrs) | 30.3 ± 5.88 | 31.4 + 5.8 | 29.6 ± 5.9 **[a] |
| BMI (kg/m$^2$) § | 28.8 ± 7.46 | 31.7 ± 7.0 | 26.7 ± 7.1 *** |
| Obesity (BMI >30 kg/m$^2$) (%) | 38.0 | 60.6 | 22.0 *** |
| Current smokers (%) | 18.7 | 15.2 | 19.9 |
| Parity | 1.1 ± 1.18 | 1.2 ± 1.18 | 1.0 ± 1.18 |
| Ethnicity (%) | | | |
| European | 86.9 | 86.0 | 87.6 |
| South Asian | 9.3 | 11.2 | 8.0 |
| Afro-Caribbean | 1.2 | 0.7 | 1.5 |
| Other | 1.2 | 1.4 | 1.0 |
| Gestation of GTT (weeks)[b] | 26.6 ± 3.95 | 26.4 ± 4.40 | 26.8 ± 3.10 |
| Mean fasting glucose (mmol/l) § | 4.9 ± 1.01 | 5.2 ± 1.15 | 4.4 ± 0.39 *** |
| Mean 2hr glucose (mmol/l) § | 7.5 ± 1.94 | 8.7 ± 1.26 | 5.6 ± 1.13 *** |
| Gestation of B12 bloods (weeks) | 26.9 ± 5.3 | 28.0 ± 4.3 | 26.2 + 5.7 ** |
| Vitamin B12 (pmol/l) § | 187.5 (146.9, 235.4) | 169.0 (140.2, 217.7) | 195.6 (157.9, 244.6) ** |
| Vitamin B12 deficiency (<150pmol/l), n (%) | 90 (26.2) | 46 (32.2) | 44 (21.9) * |
| Serum folate (nmol/L) § | 21.3 (14.0, 34.4) | 21.5 (13.5, 34.5) | 20.8 (14.5, 34.4) |
| Serum folate deficiency (<7nmol/l), n (%) | 5 (1.5) | 3 (2.1) | 2 (1.0) |
| Folic acid supplements taken (%) | 91.4 | 90.9 | 91.5 |

Continuous variables are mean ± SD (or median (IQR)), categorical variables are percentages; a: p-value as compared to GDM group, *p<0.05, **p<0.01, ***p<0.001; b: GTT results available in 90 / 201 (44.8%) of no-GDM women; §: Log-transformed for statistical comparison, GDM: gestational diabetes mellitus, BMI: body mass index, GTT: glucose tolerance test

For the whole cohort, the mean gestation of serum vitamin B12 and folate measurements was at 26.9 weeks and GTT was at 26.6 weeks. B12 levels were lower in women with GDM (169.0 vs. 195.6 pmol/l, $p<0.001$) and a significantly higher proportion of women with GDM had B12 insufficiency compared to no-GDM (Table 5.1). Folate deficiency was rare and 91% of the whole cohort was taking folate supplements. Serum folate levels were not different in the two groups.

### 5.3.2. Vitamin B12, folate status and maternal BMI and GDM

Women with B12 insufficiency had higher 1st trimester BMI than those without (30.9±7.56 vs. 28.0±7.30 kg/m$^2$, $p<0.05$). After adjusting for age, parity, ethnicity, smoking status and gestation of blood tests, BMI was a significant negative predictor of B12 (β coefficient -0.21; 95% CI -0.47, -0.13; p=0.001) whilst serum folate showed a positive association with B12 (Table 5.2, Figure 5.1). BMI was also negatively associated with serum folate after adjustment although the strength of association was weaker (β coefficient -0.12; 95% CI 0.00, 0.33; p=0.05). Third trimester vitamin B12 insufficiency was additionally associated with a 2.4 times higher odds of first trimester obesity (Table 5.3).

**Table 5.2.** Predictors of vitamin B12 and folate

| Variables | Serum B12§ β-coefficient | p-value | Serum folate § β-coefficient | p-value |
|---|---|---|---|---|
| Age | - | NS | 0.32 | <0.001 |
| Parity | - | NS | -0.24 | <0.001 |
| BMI § | -0.21 | 0.001 | -0.12 | 0.05 |
| Ethnicity | - | NS | - | NS |
| Smoking | - | NS | - | NS |
| Gestation of B12/folate bloods | - | NS | -0.28 | <0.001 |
| Serum B12 § | | | 0.12 | 0.05 |
| Serum folate § | 0.23 | <0.001 | | |
| Folic acid supplements | - | NS | - | NS |

§Log-transformed for statistical calculations; NS: non-significant

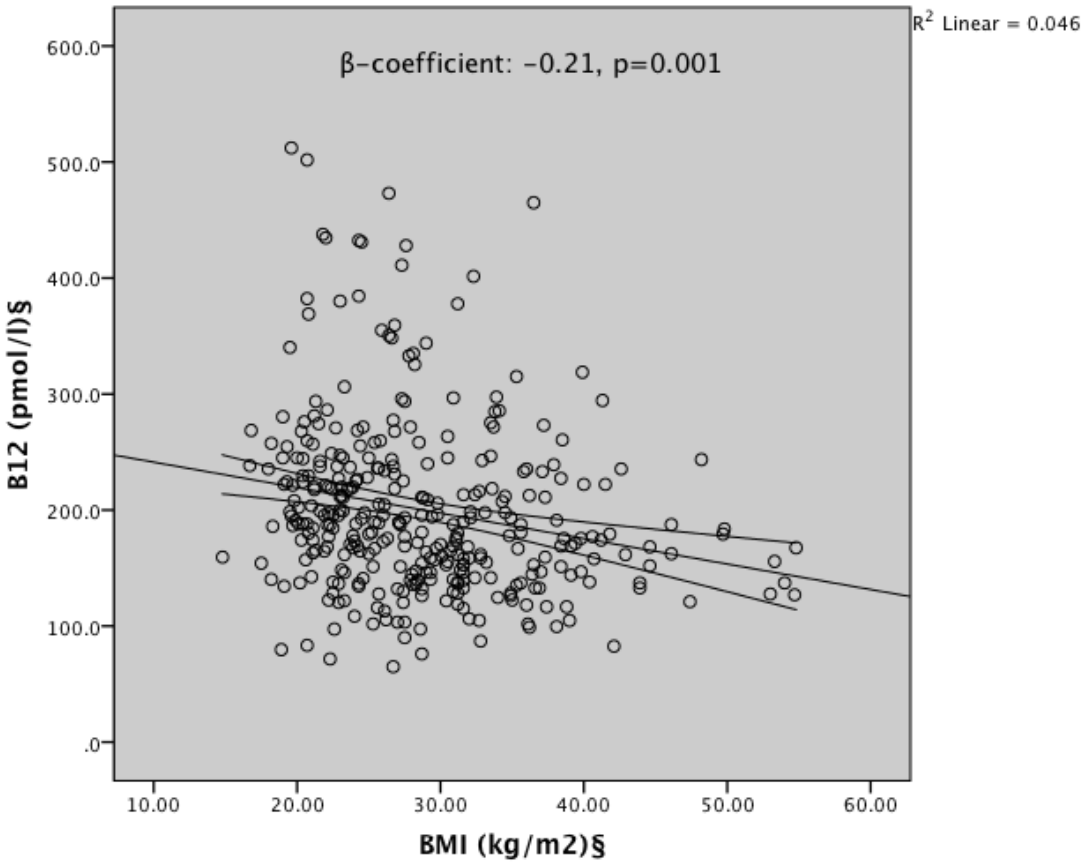


**Figure 5.1.** Scatterplot of correlation between BMI and serum B12. §Log-transformed for statistical comparisons; Regression model included age, parity, ethnicity, smoking, gestation of bloods, folic acid supplements and serum folate

B12 deficient women were at 2.59-times higher odds of having a diagnosis of GDM after adjusting for age, parity, ethnic origin, smoking, gestation of bloods and serum folate (Table 5.3). The effect size was weaker when maternal BMI was added into the model (aOR 2.05, p=0.04). Folate deficiency was not significantly associated with risk of GDM. There was also no association seen between folate thresholds and obesity.

**Table 5.3.** Relationship of maternal B12 and folate with obesity and gestational diabetes

| | n (%) | Obesity, n(%) | GDM, n(%) |
|---|---|---|---|
| *Vitamin B12 deficiency* | | | |
| Yes | 90 | 44 (49.4) | 46 (51.1) |
| No | 254 | 86 (34.0) | 97 (38.2) |
| Model 1 OR (95% CI)[a] | | 2.40 (1.31, 4.40) | 2.59 (1.35, 4.98) |
| adjusted p | | 0.004 | 0.004 |
| Model 2 OR (95% CI)[b] | | N/A | 2.05 (1.03, 4.10) |
| adjusted p | | N/A | 0.042 |
| *Folate deficiency* | | | |
| Yes | 5 | 4 (80.0) | 3 (60.0) |
| No | 332 | 125 (37.9) | 139 (41.9) |
| Model 1 OR (95% CI)[a] | | 6.29 (0.48, 82.79) | 1.93 (0.17, 22.23) |
| adjusted p | | NS | NS |
| Model 2 OR (95% CI)[b] | | N/A | 0.89 (0.07, 11.38) |
| adjusted p | | N/A | NS |

Table showing the proportions and odds ratio of obesity and development of GDM according to thresholds of B12 and folate (reference categories are 'No B12 / folate deficiency'); [a]: Model 1 adjusted for age, parity, ethnic origin, smoking, gestation of bloods and serum folate (or B12, respectively); [b]: as for Model 1 plus gestational BMI; N/A: not applicable; NS: non-significant.

### 5.3.3. Vitamin B12 and folate and birth outcomes

Birth outcome data were available in 335 women (97% of total cohort) and one baby born at less than 32 weeks gestation was excluded from this analysis. 54.5% of the babies were male and mean birthweight was 3353g. GDM women delivered 10 days earlier than no-GDM women and their mean offspring birthweight was 180g lower (3250 vs. 3428g, $p<0.01$) (Table 5.4). Due to the likely confounding effects of treatment in GDM women, the relationship between maternal B12 and folate and birth outcomes were analysed only in no-GDM women (Table 5.5). Women in the lowest quartile of B12 had higher rates of macrosomic babies compared to the highest quartile (22.9% vs. 8.0%). After adjustment for age, parity, ethnicity, smoking, serum folate, gestation of B12 bloods and newborn sex and gestational age, the relative risk (RR) of fetal macrosomia was higher in women in the lowest quartile (RR 5.26, 95% CI 1.26, 21.91, $p=0.02$). The significance was attenuated when gestational BMI was added to the model. A similar trend for the risk of LGA was observed although the result did not reach statistical significance. There was no association between B12 thresholds and outcomes of LBW or SGA. The impact of serum

folate on fetal macrosomia showed the reverse pattern for all these outcome measures. Women in the highest quartile of folate had significantly higher risk of fetal macrosomia compared to those in the lowest quartile (RR 4.99, 95% CI 1.15, 21.62, p=0.03), which remained significant after adjusting for maternal BMI (RR 6.60, 95% CI 1.42, 30.71, p=0.02).

**Table 5.4** Birth outcomes of offspring according to maternal GDM status

| Variables | Total | GDM | No GDM |
|---|---|---|---|
| Number (%) | 334 (100) | 141 (42.2) | 193 (57.8) |
| Mean fasting glucose (mmol/l) § | 4.9 ± 1.01 | 5.2 ± 1.15 | 4.4 ± 0.39 ***[a] |
| Mean 2hr glucose (mmol/l) § | 7.5 ± 1.94 | 8.7 ± 1.26 | 5.6 ± 1.13 *** |
| Birthweight (g) | 3353 ± 528 | 3250 ± 411 | 3428 ± 588** |
| Sex, n (%) male | 182 (54.5) | 76 (53.9) | 106 (54.9) |
| Gestation at birth (days) | 274 ± 10.7 | 268 ± 6.4 | 278 ± 11.3 *** |
| Macrosomia, n (%) | 42 (12.6) | 8 (5.7) | 34 (17.6)** |
| LGA, n (%) | 68 (20.4) | 29 (20.6) | 39 (20.2) |
| Low birthweight, n (%) | 14 (4.2) | 5 (3.5) | 9 (4.7) |
| SGA, n (%) | 19 (5.7) | 5 (3.5) | 14 (7.3) |

Continuous variables are mean ± SD (or median (IQR)), categorical variables are percentages. §: log-transformed for statistical comparison
a: p-value as compared to GDM group, *p<0.05, **p<0.01, ***p<0.001

**Table 5.5** Relationship between maternal B12 on birth outcome measures in no-GDM women.

| | n | Range of values (pmol/l) | Macrosomia, n (%) | LGA, n (%) | LBW, n (%) | SGA, n (%) |
|---|---|---|---|---|---|---|
| Vitamin B12 (quartiles) | | | | | | |
| 1 | 48 | 71.6, 157.2 | 11 (22.9) | 12 (25.0) | 1 (2.1) | 4 (8.3) |
| 2 | 48 | 158.7, 195.6 | 10 (20.8) | 12 (25.0) | 2 (4.2) | 2 (4.2) |
| 3 | 47 | 196.3, 244.3 | 9 (19.1) | 10 (21.3) | 3 (6.4) | 3 (6.4) |
| 4 | 50 | 245.0, 512.2 | 4 (8.0) | 5 (10.0) | 3 (6.0) | 5 (10.0) |
| Relative risk (95% CI)[a] | | | 5.26 (1.26, 21.91) | 3.18 (0.96, 10.56) | 0.10 (0.002, 5.75) | 1.35 (0.28, 6.47) |
| p[b] | | | 0.02 | 0.06 | 0.27 | 0.71 |
| p[c] | | | 0.05 | 0.13 | 0.37 | 0.52 |
| Folate (quartiles) | | | | | | |
| 1 | 44 | 4.5, 14.3 | 5 (11.4) | 7 (15.9) | 4 (9.1) | 4 (9.1) |
| 2 | 47 | 14.5, 20.6 | 7 (14.9) | 9 (19.1) | 1 (2.1) | 2 (4.3) |
| 3 | 48 | 20.8, 34.2 | 11 (22.9) | 10 (20.8) | 1 (2.1) | 3 (6.3) |
| 4 | 48 | 34.4, 45.3 | 10 (20.8) | 12 (25.0) | 3 (6.3) | 5 (10.4) |
| Relative risk (95% CI)[a] | | | 4.99 (1.15, 21.62) | 2.32 (0.74, 7.34) | 0.21 (0.01, 9.64) | 1.52 (0.26, 8.93) |
| p[b] | | | 0.03 | 0.15 | 0.42 | 0.64 |
| p[c] | | | 0.02 | 0.06 | 0.41 | 0.90 |

[a]: Relative risk of birthweight outcome in quartile 1 vs. quartile 4 of B12 and quartile 4 vs. quartile 1 of folate; [b]: adjusted for age, parity, ethnic origin, smoking, gestation of bloods and serum folate (or B12, respectively), plus sex and gestational age for macrosomia and LBW; [c]: as for Model b plus gestational BMI

### 5.4. Discussion

Our study, although retrospective in nature, showed three key findings. Firstly, it is the first to show that low B12 status in pregnancy is associated with higher risk of GDM in a UK population. Secondly, higher first trimester BMI was an independent predictor of later B12 insufficiency. Thirdly, low B12 levels were associated with macrosomia in the subgroup of no-GDM women, which seems to be partly mediated by maternal BMI.

The only other study that examined the link between B12 and GDM by *Krishnaveni et al* was in an Indian cohort (Krishnaveni et al. 2009). The magnitude of association found in that study was similar to ours but the significance was lost after adjusting for maternal BMI. In our study, although the effect size was reduced when adjusted for BMI (aOR 2.59 vs. 2.05; Table 3), the significance persisted suggesting a potential independent effect of B12. Higher numbers of women with GDM in our cohort and a 'case-control' design might explain the larger effect size. The recent finding by Knight et al, albeit in no-GDM women, also supports the inverse link between B12 levels and insulin resistance in pregnant White Caucasian women (B. A. Knight et al. 2015). Indeed, higher insulin resistance in the context of low B12 has been shown by other authors in obese adolescents(M. Ho et al. 2014), non-pregnant adults (Baltaci et al. 2013; Kaya et al. 2009) as well as in women with polycystic ovarian syndrome (Kaya et al. 2009). Prospective longitudinal studies are needed to investigate whether the presence of low B12 status in early pregnancy independently increases the risk of incident GDM.

We found no relationship between folate levels and risk of obesity or GDM but in the study by *Krishanveni et al*, the incidence of GDM increased with increasing folate concentrations in the subgroup of women with B12 deficiency (5.4%, 10.5% and 10.9% from lowest to highest tertile respectively, p for trend = 0.04).

The aetiology of the inverse relationship between B12 and BMI found in our study is an intriguing one. While confounding factors such as dietary habits, socioeconomic status and hemodilution may be present, other studies that have corrected for these still show an independent link between B12 and BMI (B. A. Knight et al. 2015; Krishnaveni et al. 2009). Interestingly, frying and roasting of meat products reduces

the bioavailability of B12 by 20-40% (United States Department of Agriculture 2007), so higher consumption of processed foods may increase the risk of both B12 insufficiency and metabolic diseases. Additionally, B12 has been shown to be negatively associated with other markers of obesity such as triglycerides (Adaikalakoteswari et al. 2014), blood pressure (Karatela and Sainani 2009) and the metabolic syndrome (Guven et al. 2005), which lends support to a possible pathological association between them. In one trial, supplementation of B12 and folate in adults with metabolic syndrome improved insulin resistance by ameliorating endothelial dysfunction, providing further insight into how these conditions may be linked (Setola et al. 2004). Further studies are needed to determine the direction of association and a potential reverse causality.

This is the first study that has demonstrated a relationship between maternal B12 and macrosomia, which seem to be mediated in part by maternal obesity. We demonstrated this only in no-GDM women as the treatment of GDM is a major confounder for macrosomia. Unfortunately, we did not have adiposity measures or a bigger sample size to assess the interactions between B12 status and maternal BMI/adiposity with offspring size and adiposity.

The rates of B12 insufficiency observed in our no-GDM population was similar to that was observed in Knight et al (B. A. Knight et al. 2015) (22% vs. 20%) suggesting that such higher rates of insufficiency are not limited to Indian populations (Sukumar et al. 2016). It must be noted that a fall in B12 during pregnancy may be physiological due to a decrease in the fraction bound to inactive haptocorrin (Koebnick et al. 2002), but the evidence is equivocal with regards to whether there is also a fall in the active form, holotranscobalamin (Greibe et al. 2011; Murphy et al. 2007). In the absence of specific cut-off values to define B12 deficiency in pregnancy, we used the non-pregnant reference range (<150pmmol/l). It is noteworthy that associations with adverse maternal metabolic outcomes (Krishnaveni et al. 2009) and elevation in Hcy during pregnancy (Guerra-Shinohara et al. 2004) were found by other authors at B12 thresholds similar to this.

It was reassuring to see that folate deficiency was rare, albeit in this selected hospital-based cohort. However, the combination of low B12 and high folate has been shown

to be associated with lower neonatal birthweight (Gadgil et al. 2014) as well as central adiposity and insulin resistance in 6-year old offspring (C. S. Yajnik et al. 2008). Whilst our sample size was not large enough to do detailed subgroup analysis, we observed that women in the lowest quartile of B12 and highest quartile of folate had similar risks of macrosomia (aRR of 5.3 and 4.99; Table 4). Therefore, it is possible that the women with such a B12-folate imbalance are particularly at high risk of having larger babies. This phenomenon (high folate / low B12), is increasingly common in populations with mandatory folic acid fortification such as USA and Canada (Ray et al. 2003; Wyckoff and Ganji 2007) and is related to adverse clinical outcomes in the elderly population (Morris et al. 2007).

Although we have identified associations between B12, maternal obesity, risk of GDM and fetal macrosomia, our study does not prove causation or the direction of relationship between these factors. Some of the important limitations were that this was a single-centre, retrospective study involving pregnant women attending a hospital clinic. Therefore it was not possible to obtain early pregnancy B12/folate levels. We adjusted for gestation of bloods in all the regression analysis, to reduce some of the bias due to longitudinal changes in B12 during pregnancy. We did not have markers of adiposity, and therefore it was not possible to study the potential differential association of low B12 status with obesity and adiposity in pregnant women as well as their offspring. Lack of functional measures of B12 insufficiency, such as Hcy and methylmalonic acid (MMA), or holotranscobalamin, which is the active fraction of B12 available for uptake by tissues, limits the ability to study thresholds of B12 sufficiency during pregnancy and should be measured in future studies. Additionally, there are variables which were not measured in this study that have been shown to affect maternal B12 levels (eg. socioeconomic status and inter-pregnancy intervals), which should be accounted for in further prospective studies.

### 5.5. Conclusion

This study shows, for the first time in a UK population, that B12 deficiency in pregnancy is common particularly in obese women, is independently associated with GDM and may contribute to macrosomia. As the prevalence of maternal obesity and GDM is rapidly increasing, our findings warrant longitudinal cohort studies to understand the interplay between B12 and these outcomes. If early pregnancy B12

status found to be independently predictive of incident GDM, such findings could potentially offer simple intervention to improve the metabolic health of pregnant women and their offspring.

# Chapter 6

# Vitamin B12 status in women of child-bearing age in the UK and its relationship with national nutrient intake guidelines: Results from two National Diet and Nutrition Surveys

## 6.1. Introduction

As reviewed in Chapters 3-5, it is well known that vitamin B12 (B12) necessary for the synthesis of DNA, RNA, lipids and protein and an essential step in this process is the conversion of homocysteine to methionine, Therefore, a deficiency of either B12 or folate leads to increased homocysteine (Hcy) which can have significant clinical implications such as cardiovascular disease and atherosclerosis in adults (Wald et al. 2002; Zhou and Austin 2009). Vitamin B12 insufficiency was previously perceived to be a problem affecting the elderly, due to malnutrition or intrinsic-factor mediated malabsorption (Stabler and Allen 2004) and has been related to anaemia, dementia and cognitive dysfunction (Clarke et al. 1998; Refsum and Smith 2003).

Although hyperhomocysteinaemia (most commonly due to B12 or folate deficiency) has been identified as an independent risk factor for atherosclerotic vascular disease (Boushey et al. 1995; Homocysteine Studies 2002), a systematic review showed no definite association between B12 insufficiency in adults and composite cardiovascular endpoints (Rafnsson et al. 2011).

One of the most serious effects of low B12 and folate during early pregnancy is the development of neural tube defects and the possible mechanisms for this were discussed in Chapter 3. Folic acid supplementation is effective in reducing the risk of NTD by over 40% (de Jong-van den Berg 2008) but because more than half of pregnancies are unplanned, mandatory folic acid fortification of wheat flour and cereal products was introduced in North America in 1997 and many other parts of the world in the early 2000s (L. B. Finer and Zolna 2011). This resulted in halving of NTD due to folate deficiency in 10 years (Centers for Disease Control and Prevention 2004). However, the number of NTD attributable to B12 deficiency has tripled in this time (Ray et al. 2007).

The implications of B12 deficiency during pregnancy as a risk factor for obesity and gestational diabetes (GDM) as well as associations with extremities of birthweight have been discussed in Chapters 4 and 5. Maternal hyperhomocysteinaemia has been linked to early pregnancy losses, pre-eclampsia and small for gestational age babies

(Hogeveen et al. 2012; Nelen et al. 2000; Vollset et al. 2000). Although the exact mechanisms are not known, some of these effects may be mediated by vascular compromise to the fetus and insufficient placental development which occurs in the early embryonic period (Koukoura et al. 2012).

Therefore, it is important to optimise the nutrient status of women in the preconceptional period. In the UK, there is no national programme for mandatory folic acid fortification but all women planning a pregnancy are advised to take at least 400mcg of folic acid supplements until the end of their first trimester. However there is no evidence or guidelines for the use of B12 supplements in pre-conception or early pregnancy so it is necessary to refer to the national recommended nutrient intake (RNI) guidelines to inform us about the adequacy of this micronutrient's intake. Thus far, there have been no studies in the UK that assessed the B12 intake or circulating levels in women of reproductive age prior to conception.

The UK Department of Health (DOH) has stipulated that the RNI for B12 and folate in adults is 1.5mcg and 200mcg per day respectively, with no different recommendations for women (pregnant or otherwise) and the elderly (COMA (Committee on Medical Aspects of Food Policy) 1991). These recommendations were published in 1991 and state that the RNI for B12 "represent the level of intake considered likely to be sufficient to meet the requirements of 97.5% of the group" (Whitehead 1992). The evidence for this RNI is from reports in the 1970's, which estimated the average requirement to prevent diet-related B12 deficiency and megaloblastic anaemia (S. J. Baker and Mathan 1981; Cooper and Lowenstein 1966; EFSA NDA Panel (EFSA Panel on Dietetic Products 2015). B12 deficiency in these studies was arbitrarily defined as <150pmol/L without use of any functional markers of B12 deficiency (Doets et al. 2012; Institute of Medicine 1998).

Based on the above, it is obvious that there is little contemporary data to support how accurately the recommended intakes correlate with serum values of B12 and functional indicators such as Hcy. The primary aim of this study is to determine the serum B12, folate and Hcy status of women of child-bearing age in the UK and to

assess the correlation between estimated B12 intake and blood concentrations of B12 and Hcy utilising the data from two British National Diet and Nutrition Survey (NDNS) in 2000/01 and 2008/12. The secondary aim is to compare the nutritional intake between the two NDNS cohort to provide more recent data on B12 intake.

## 6.2. Methods

### 6.2.1. Study design and justification

Since the aim of this study was to evaluate B12 and folate intake among women of child-bearing age in the UK, a national survey which collected this information and additionally measured biomarker levels was an ideal way to meet the objectives. The NDNS has publically available datasets which were downloaded and statistical analysis done by myself to answer the study questions.

Data collected from the two British NDNS between July 2000 and June 2001 and 2008-2012 were collected. The surveys provide detailed quantitative assessment of nutritional status and laboratory results of the participants. The methods used in the survey have previously been described in detail (Bates et al. 2014; Henderson et al. 2002; Henderson et al. 2003). The samples were made up of randomly selected adults aged 19-64 years living in private households who were representative of the UK adult population. Their selection was done by a multi-stage, stratified, probability sampling with postal sectors as first stage units. If there were more than one adult in the same household one was selected randomly. Women who were pregnant or breastfeeding were excluded.

### 6.2.2. Participant selection

The selection of the participants included in our analysis is provided in Figures 6.1 A-B. For the first survey (2000/01), the fieldwork was carried out over a 12-month period, with respondents being surveyed over four three-month periods to account for seasonal variations in nutritional behavior and content. Out of 3,704 potentially eligible adults identified for the study, 299 women between 19 and 39 years of age (known as women of ‘child-bearing age’ for the purpose of our analysis) with

complete dietary and biomarker information were included in the final analysis. In an independent study (Skinner CJ 2002), no evidence of  was found of non-response bias in this NDNS data. Although 'childbearing years' is usually defined as 15 to 44 years (Office for National Statistics 2016), our study was limited to women from 19 to 39 years because this was a national survey of adults aged 19 to 64 years so teenagers aged 15 to 18 were not included. Advancing maternal age is an independent risk factor for NTD (De Marco et al. 2011) so it is possible that older women have different B12 and folate requirements in periconception or pregnancy to prevent such complications. Therefore the upper age limit was set as 39 years.

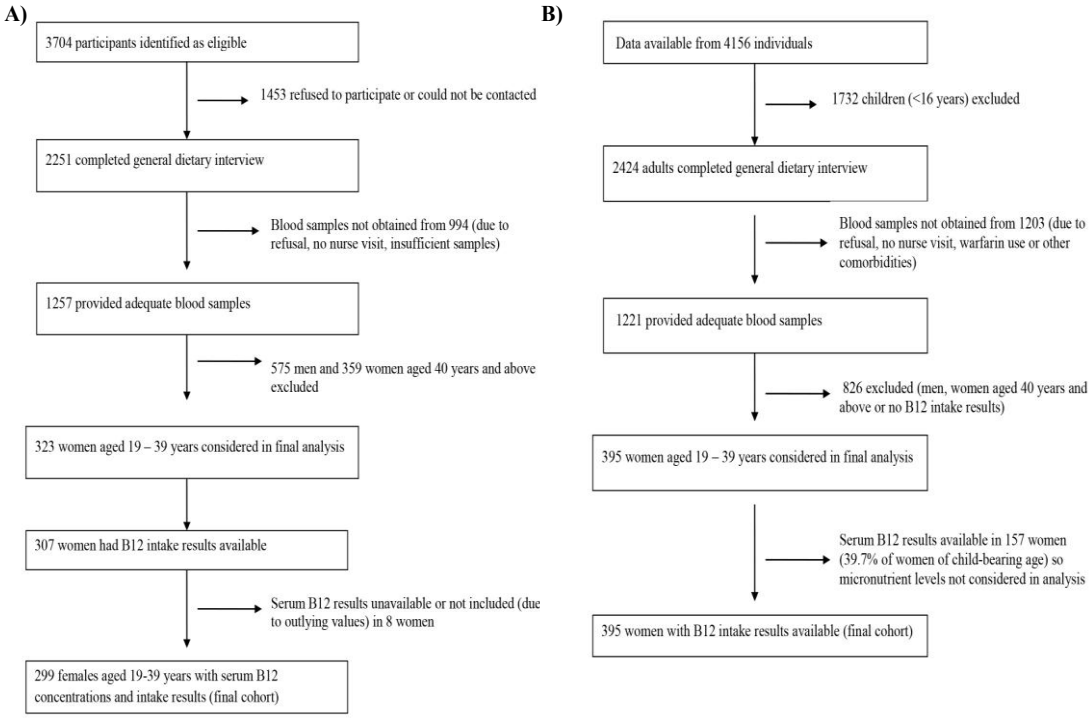


**Figure 6.1** Flow diagram illustrating the selection of the final cohort in the **A)** NDNS 2000/01 and **B)** NDNS 2008/12 survey

The second survey (2008/12) consisted of 2424 adults of whom 395 were women of child-bearing age. Unfortunately, blood samples were only obtained from 157 of these women (i.e. 39.7% of all females aged 19 to 39 years). The main reasons for this low return of blood samples were "no nurse visit", "participant refused" and "blood sample inapplicable". Due to the potential bias from a sample of women not representative of the larger NDNS cohort, we only included and compared the micronutrient intakes from the two surveys to assess the secular trend.

### 6.2.3. Assessment of dietary intake

This has been described in detail elsewhere (Henderson et al. 2002). Briefly, dietary assessment for each participant was done by a two-stage process: 1) two face-to-face interviews using computer assisted personal interviewing methods and 2) a seven-day dietary record using weighed food diaries. Each participant was provided with a set of Soehnle Quanta digital food scales and two recording diaries (for use at home and outside). Additional information was obtained about the use of medicines, vitamin and mineral supplements. Information provided in the food diaries was later used to determine nutrient intakes by linking to the Food Standards Agency nutrient database, which holds details for 56 nutrients for each of 6000 food codes (Henderson et al. 2002).

### 6.2.4. Laboratory methods

Venous blood samples were taken at the non-fasting state by trained phlebotomists in participants' homes. Serum folate and B12 measurements were performed on the Abbott IMx semi-automated analyser, which uses Microparticle Enzyme Immunoassay (MEIA) technology (z). Quality control consisted of an internal pooling of serum samples with each run for use as a drift control, and an external quality assessment by the United Kingdom National External Quality Assessment Service (NEQAS). Outlying results were defined as serum B12 level greater than 1000pmol/L and serum folate greater than 60nmol/L and were excluded. Plasma homocysteine measurements were performed by the Abbott IMx assay on the IMx analyser. Quality control consisted of participation in an international external quality assessment scheme based in Denmark (Moller et al. 1997) and by the manufacturer's QV samples.

### 6.2.5. Statistical analysis

Statistical analysis was performed with SPSS version 22.0 (IBM Corp Released 2013). For continuous variables (e.g. mean B12/folate intakes and levels between RNI threshold groups) the Students t-test and for categorical variables (e.g. B12 and folate deficiency rates), the chi-squared test for independence or the Fisher's exact test were used. Stepwise multiple linear regression analysis with each of serum B12, folate and plasma homocysteine as the dependent variable was done, with predictors were

entered or removed following the criteria: Probability-of-F-to-enter ≤0.050, Probability-of-F-to-remove ≥0.100. The regression models included the following covariates: age, body mass index (BMI), total cholesterol: high density lipoprotein ratio, alcohol intake, smoking status, oral contraceptive use, vegan/vegetarian, serum folate and where appropriate daily folate intake (with supplements), daily B12 intake (with supplements) and serum B12 levels. Race was not included in the analysis as the majority of the participants (95%) were white. To determine the trend of B12 and Hcy across the spectrum of B12 intakes, we divided the cohort into quintiles of B12 intake. One-way ANOVA with Tukey's post-hoc test was applied to compare the serum levels against the lowest quintile. Logistic regression was then done for incremental B12 intake thresholds of 0.25mcg to determine predictors of B12 deficiency and hyperhomocysteinaemia. In order to estimate the prevalence of inadequate B12 intake in our population and observe how this was related to abnormal biomarker levels, we utilized the Estimated Average Requirement (EAR) cut-point method (Beaton 1994). The EAR was calculated as 1.25mcg/day based on a coefficient of variation of 10% below the UK RNI (Doets et al. 2012).

The definitions for micronutrient deficiencies were as follows: serum B12 ≤150pmol/L (Carmel 2011); serum folate ≤10nmol/L (WHO 2008) and red cell folate <350nmol/L (WHO 2008). The upper limit of normal for plasma Hcy in non-pregnant adults aged 15 to 65 has been variably defined as 12umol/L in non-pregnant adults with folic acid fortification or supplementation and 15umol/L in those without fortification or supplementation, which applies to our population (Refsum et al. 2004). However, Hcy levels above 10.7 umol/L in women during pre-conception has been associated with pre-eclampsia, prematurity and very low birthweight infants if they become pregnant (Vollset et al. 2000). In addition, other experts recommend <12umol/L for all adults (Fokkema et al. 2001). Therefore, I have presented results for both 12umol/L and 15umol/L and stated these clearly throughout.

### 6.2.6. Ethics approval

Ethical approval was obtained from a multi-centre Research Ethics Committee and the Oxfordshire A Research Ethics Committee for the 1st and 2nd surveys respectively

and all Local Research Ethics Committees covering areas where the fieldwork was conducted.

## 6.3. Results

### 6.3.1. Overall study parameters

From the NDNS 2000/01 cohort, 299 women of child-bearing age had complete dietary and serum micronutrient level results. The demographics and clinical characteristics of these women are shown in Table 6.1. Their mean age was 31.6 years and BMI 25.3kg/$m^2$. To determine how the B12/folate intakes women of child-bearing age compared to older women from the same NDNS survey, we analysed these parameters in women aged 40-64 years. The younger women consumed significantly less B12 and folate (median 3.83 vs. 5.16 and 237 vs. 279 mcg/day respectively, $p<0.001$ for both) and consisted of more vegetarians/vegans (8.7 vs. 4.2%, $p<0.05$) and less B12 supplement users (10.4 vs. 16.2%, $p<0.05$). Folic acid supplement use was the same (data not shown).

**Table 6.1** Demographics and B12 and folate intakes of women of child-bearing age (NDNS 2000/01 cohort)

| **FEMALE 19-39 Years** | **All subjects (n=299)** |
|---|---|
| Age (yrs) | 31.6 ± 5.7 |
| BMI (kg/$m^2$) | 25.4 ± 5.2 |
| Obesity, n (%) | 46 (15.7) |
| Current smokers, n (%) | 117 (39.1) |
| Regular alcohol drinkers, n (%) | 267 (89.3) |
| Oral contraceptive pill use, n (%) | 103 (34.4) |
| Ethnicity, n (%) | |
| White | 283 (94.6) |
| Afro-Caribbean | 3 (1.0) |
| Asian | 8 (2.7) |
| Other | 5 (1.7) |
| Vegetarians, n (%) | 26 (8.7) |
| B12 supplement users, n (%) | 31 (10.4) |
| B12 intake, diet only (mcg/day) | 3.82 (2.75, 5.02) |
| B12 intake, diet + supplements (mcg/day) | 3.83 (2.82, 5.20) |
| Folic acid supplement users, n (%) | 32 (10.7) |
| Folate intake with supplements (mcg/day) | 237 (177, 315) |

Continuous variables are mean ± SD or median (IQR)
Categorical variables are n(%)

### 6.3.2. B12, folate and homocysteine status in women of child-bearing age

The blood levels of B12, folate and homocysteine in women of child-bearing age and according to categories of UK RNI for B12 intake (adequate/inadequate) are given in Table 6.2. The median serum B12 concentration was 241pmol/l. Overall, 12% of women were B12 deficient (<150pmol/L), despite the median B12 intake of the deficient women being nearly two times the UK RNI (2.96mcg/day) (data not shown). 3.7% of the surveyed population had both B12 <150pmol/L and Hcy >12umol/L, with a significantly higher proportion having the combination of abnormalities when their estimated was lower than the UK RNI (9.1% vs. 3.5%, p=0.001). There is evidence that B12 levels <258 pmol/l may be indicative of B12 deficiency in certain individuals with concomitant elevation of Hcy and MMA (Lindenbaum et al. 1994). 44.0% of women had B12 levels in this borderline range of 150 – 258 pmol/l. In this subgroup, mean Hcy levels were significantly higher than the group with B12 >258pmol/l (10.4 vs. 9.2umol/L, p=0.02) despite similar folate levels (21.4 vs. 22.0nmol/L, p=NS).

The plasma Hcy concentrations were higher in the lower intake group (11.9 vs. 9.2umol/L, p<0.05) although hyperhomocysteinemia (Hcy >12umol/L) was present in 20% of women with apparently adequate B12 intake (Table 6.2). Serum and red cell folate deficiency rates were 6.1% and 4.4% respectively in the whole population. There were no differences in the folate deficiency rates between above and below the UK RNI B12 intake groups. 34.4% of the women were taking the oral contraceptive pills and their B12 values were lower than those who were not (median 211.5 vs. 267.5 pmol/L, p<0.001).

8.7% of women in the whole cohort were vegetarian or vegan and their median dietary intake of B12 was non-significantly lower than non-vegetarians (2.95 vs. 3.87 mcg/day) while folate consumption in the former group was higher (Table 6.3). Vegetarians had lower serum B12 concentrations (median 192 vs. 248pmol/L, p<0.01) but their folate or Hcy concentrations did not vary significantly.

**Table 6.2** Comparison of B12, folate and plasma homocysteine concentrations in women according to UK RNI for vitamin B12 intake

| FEMALE 19-39 Years | All subjects | UK RNI (mcg/day) | | P[a] |
|---|---|---|---|---|
| | | <1.5 | ≥1.5 | |
| Number (%) | 299 (100) | 11 (3.7) | 288 (96.3) | |
| B12 intake, diet only (mcg/day) | 3.82 (2.75, 5.02)[b] | 1.29 (0.64, 1.46) | 3.86 (2.86, 5.09) | <0.001 |
| B12 intake, diet + supplements (mcg/day) | 3.83 (2.82, 5.20) | 1.29 (0.98, 1.46) | 3.92 (2.88, 5.32) | <0.001 |
| Serum B12 (pmol/L) | 241 (188, 324) | 169 (153, 256) | 244 (189, 325) | 0.05 |
| B12 deficiency (<150pmol/L), n (%) | 36 (12.0) | 2 (18.2) | 34 (11.8) | NS |
| Serum folate (nmol/l) | 19.5 (14.1, 26.7) | 14.3 (13.6, 21.3) | 19.7 (14.2, 27.0) | NS |
| Serum folate deficiency (<10nmol/L), n (%) | 18 (6.1) | 0 (0) | 18 (6.3) | NS |
| Red cell folate (nmol/L) | 584 (473.9, 750.6) | 460 (372, 739) | 585 (478, 751) | NS |
| Red cell folate deficiency (<350nmol/L), n)%) | 13 (4.4) | 2 (18.2) | 11 (3.8) | NS |
| Homocysteine (umol/L) | 9.4 (9.1, 9.8) | 11.9 (9.6, 14.3) | 9.2 (7.8, 11.4) | <0.05 |
| High Hcy (>12 umol/L), n (%) | 62 (21.2) | 5 (50) | 57 (20.1) | <0.05 |
| High Hcy (>15 umol/L), n(%) | 24 (8.2) | 2 (20) | 22 (7.8) | NS |

a: Comparison between lower and higher B12 intake groups. For categorical variables, Student's t-test was used (after log-transformation); for continuous variables Fisher's exact test was used

b: Median, 25th -75th centile in parentheses (all such values)

NS: not significant

**Table 6.3** Biochemical indices in vegetarian and vegan women of child-bearing age compared to non-vegetarians

| | **Vegetarian / vegan** | **Non-vegetarian** | **P[a]** |
|---|---|---|---|
| Number (%) | 26 (8.7) | 273 (91.3) | NS |
| B12 supplements taken, n (%) | 5 (19.2) | 26 (9.5) | NS |
| B12 intake, diet only (mcg/day) | 2.95 (2.63, 3.96)[b] | 3.87 (2.81, 5.09) | NS |
| B12 intake, diet + supplements (mcg/day) | 3.07 (2.65, 4.37) | 3.92 (2.86, 5.30) | NS |
| Serum B12 (pmol/L) | 192.0 (138.9, 248.2) | 248.3 (190.5, 328.3) | <0.01 |
| B12 insufficiency (<150pmol/L), n (%) | 8 (30.8) | 28 (10.3) | <0.01 |
| Folic acid supplements taken, n (%) | 3 (11.5) | 29 (10.6) | NS |
| Folate intake, diet only (mcg/day) | 279.2 (234.6, 324.0) | 232.2 (169.3, 292.0) | <0.05 |
| Folate intake, diet + supplements (mcg/day) | 284.2 (234.6, 337.5) | 233.9 (175.8, 310.2) | NS |
| Serum folate (nmol/L) | 21.8 (18.5, 30.8) | 19.2 (13.8, 26.2) | <0.05 |
| Serum folate deficiency (<10nmol/L), n (%) | 1 (3.8) | 17 (6.3) | NS |
| Homocysteine (umol/L) | 9.60 (7.74, 11.90) | 9.24 (7.63, 11.46) | NS |
| High Hcy (>12 umol/L), n (%) | 5 (19.2) | 57 (21.3) | NS |
| High Hcy (>15 umol/L), n(%) | 1 (3.8) | 23 (8.6) | NS |

a: Comparison between vegetarian/vegan and non-vegetarian groups. For categorical variables, Student's t-test was used (after log-transformation); for continuous variables Fisher's exact test was used

b: Median, 25th -75th centile in parentheses (all such values)

NS: not significant

### 6.3.3. Predictors of B12, folate and homocysteine levels

There was a positive correlation between serum B12 values and daily B12 intake (Pearson's r=0.27, p<0.001) (Figure 6.2). Simple linear regression analyses of the predictors of B12, folate and homocysteine are shown in Table 6.4. After adjusting for the likely confounders, daily B12 and folic acid intakes were positive predictors of serum B12 (β = 0.28, p<0.001) and serum folate (β = 0.33, p<0.001) respectively. Along with age, serum B12, serum folate and B12 intake were independent predictors of homocysteine though it would appear that serum folate is strongest based on the value of the β coefficient.

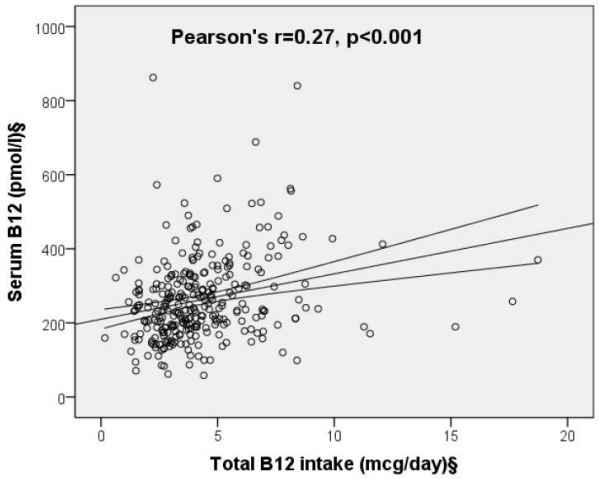


**Figure 6.2** Correlation between daily B12 intake and serum B12 values. §Log transformed for statistical comparisons.

**Table 6.4** Multiple linear regression analysis of predictors of serum B12, folate and homocysteine

| Variables | Serum B12§ | | Serum folate | | Homocysteine§ | |
|---|---|---|---|---|---|---|
| | **β-coefficient** | **p-value** | **β-coefficient** | **p-value** | **β-coefficient** | **p-value** |
| Age | - | NS | - | NS | 0.18 | 0.001 |
| BMI | - | NS | - | NS | - | NS |
| Smoking | -0.12 | <0.05 | - | NS | - | NS |
| Alcohol | - | NS | - | NS | - | NS |
| Oral contraceptive use | -0.29 | <0.001 | 0.11 | <0.05 | - | NS |
| TC:HDL ratio§ | - | NS | - | NS | - | NS |
| Vegetarian or vegan diet | -0.18 | <0.01 | - | NS | - | NS |
| B12 supplement use | - | NS | Not included | N/A | - | NS |
| Daily B12 intake§ | 0.28 | <0.001 | Not included | N/A | -0.16 | 0.001 |
| Folic acid supplement use | Not included | N/A | 0.18 | <0.01 | - | NS |
| Daily folate intake§ | Not included | N/A | 0.33 | <0.001 | - | NS |
| Serum B12§ | | | - | NS | -0.20 | <0.001 |
| Serum folate§ | - | NS | | | -0.35 | <0.001 |

§: Log-transformed for statistical comparison
-: Tested but not significant in the model
NS: Non-significant

### 6.3.4. Relationship between B12 intake and associated biomarkers

5/299 (1.7%) of women consumed below the EAR of 1.25mcg/day but none of them had B12 levels <150pmol/L. Conversely, in the 98.3% of women with 'adequate' EAR category for B12 consumption, 12.2% of them had serum levels below 150pmol/L.

In order to determine the trend of blood B12 and Hcy concentrations with increasing intakes of B12, we divided the cohort in quintiles of B12 intake. The median levels in each quintile and associated biomarker levels for B12 and Hcy are represented in Figures 6.3A-B respectively. Women in quintiles 4 and 5 (median intake 4.9 and 7.1mcg/day) had significantly higher mean B12 and lower mean Hcy concentrations than quintile 1 (291pmol/L and 322pmol/L vs. 229pmol/L; 9.0umol/L and 9.0umol/L vs. 11.4 umol/L respectively). Additionally, the Hcy levels appears to level off between quintiles 4 and 5, suggesting that increasing intakes above 5mcg/day is unlikely to provide higher B12 at the tissue level (Figure 6.3B). To confirm this, we performed logistic regression to determine the intake threshold at which the odds of hyperhomocysteinaemia would reduce significantly after correcting for confounders. Using 0.25mcg increments, a threshold of 4.75mcg/day significantly reduced the odds of Hcy>12umol/L (aOR 0.35, 95% CI 0.14-0.88). When serum B12 was added to the model, the significance was lost suggesting that the influence of dietary B12 intake on circulating Hcy is mediated through serum B12 values in a folate-replete population (data not shown).

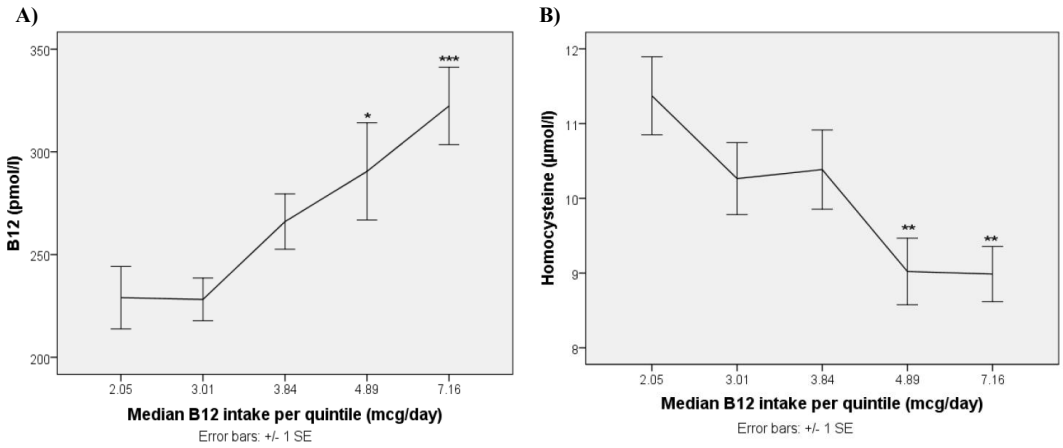


**Figure 6.3** Relationship between B12 intake in quintiles and **A)** mean serum B12 and **B)** mean plasma homocysteine concentrations. Mean±SEM values are plotted against the median B12 intake in each quintile. One-way analysis of variance (ANOVA) test used to compare the means between the quintiles (after log transformation) and Tukey's post hoc analysis done. Mean biomarker levels differed as compared with quintile 1 as follows: *p<0.05; **p<0.01; ***p<0.001.

### 6.3.5. Micronutrient intake: comparisons between NDNS 2000/01 and 2008/12 data

Comparison of the mean nutrient intake among young women between the two surveys is shown in Table 6.5. The proportion of women consuming below the UK RNI for folate increased in the more recent survey (44 vs. 33%, p<0.01) and median intake levels fell by 14% (206 vs. 235 pmol/L, p<0.001). There were no significant differences in the B12 intake values.

**Table 6.5** Comparison between folic acid and B12 intakes between the NDNS 2000/01 and 2008/11 cohorts

| Female 19-39 Years | NDNS 2000/01 Cohort | NDNS 2008/11 Cohort |
|---|---|---|
| Number | 299 | 395 |
| Folic acid supplement users, n (%) | 32 (10.7) | 12 (3.0)***[b] |
| Folate intake, diet only (mcg/day) | 234.9 (175.7, 302.7)[a] | 205.8 (162.3, 261.2)*** |
| n (%) consuming below UK RNI of folate | 99 (33.1) | 173 (43.8)** |
| B12 supplement users, n (%) | 31 (10.4) | 33 (8.4) |
| B12 intake, diet only (mcg/day) | 3.82 (2.75, 5.01) | 3.78 (2.64, 4.84) |
| n (%) consuming below UK RNI of B12 | 11 (3.7) | 24 (6.1) |

a: Median, 25th -75th centile in parentheses (all such values)
b: p-values from independent samples t-test for continuous variables (after log-transformation) or chi-square test for categorical variables
Significance: **p<0.01; ***p<0.001

### 6.4. Discussion

This study shows contemporary data of B12 and folate intakes and serum levels in population-based nutritional surveys involving women of child-bearing age, who are selected from the general UK population. The key findings are that, despite an apparent adequate daily intake of B12, a high proportion of women have B12 deficiency and hyperhomocysteinaemia.

*Optimum B12 intake and biomarker levels*

This data showed positive correlation between B12 intake and blood levels with a trend of increasing B12 concentration even in intakes up to 7mcg/day, although the corresponding reductions in Hcy levels level off around the intake of 5-7mcg/day. Additionally, the consumption of around 4.75mcg/day was independently associated with a decrease in the odds of hyperhomocysteinaemia. Our findings are supportive of the dose-response relationship between intake and blood levels of B12 found previously, which showed doubling B12 intakes would increase B12 concentrations by around 10% (Dullemeijer et al. 2013).

A daily intake of 4-10mcg has been suggested by other studies to stabilise levels of B12, Hcy and the associated MMA in adults (Bor et al. 2010; Vogiatzoglou et al. 2009) but more evidence is needed before extrapolating these figures to women of child-bearing age due to the different requirements and implications should they become pregnant. In a randomised control trial where all subjects consumed 8.6mcg/day of B12, pregnant women had higher holotranscobalamin : total B12 ratios than non-pregnant, non-lactating controls suggesting that such high intakes were required to provide sufficient supply to the developing fetus (Bae et al. 2015). More research is required to decide on the upper limit of B12 intake in women before and during pregnancy, as higher B12 intake in the 3$^{rd}$ trimester was positively associated with offspring birthweight in high BMI women (who are already at high risk of having macrosomic babies) although circulating B12 levels were not reported here (Horan et al. 2015). There is also no consistent evidence that increased B12 intake or supplementation is associated with reduction in the prevalence of subclinical B12 deficiency in adults (particularly neurocognitive decline in the elderly) (A. D. Smith

et al. 2010; A. D. Smith and Refsum 2012). Therefore, until the availability of further studies, our data calls for revision of the UK RNI (and EAR) for B12 to be at least in line with the current European recommendations (4.5mcg/day) (EFSA NDA Panel (EFSA Panel on Dietetic Products 2015).

The estimated B12 intake in pre-conceptional or women in early pregnancy, and specifically its relationship with serum levels has not been widely studied. In developing countries, over 50% of women of reproductive age do not meet the RNI's for B12 (Arsenault et al. 2013; Nguyen et al. 2014) and separate surveys from three developing countries (Turkey, Iran and China) found B12 deficiency rates of 21-23% (Abdollahi et al. 2008; Karabulut et al. 2015; Zhu et al. 2010). An Australian study on young females found a similar rate to ours (11.4%) albeit using a lower threshold of 120pmol/L (Fayet-Moore et al. 2014). As the B12 levels fall by around 10% from pre-conception to early pregnancy (Murphy et al. 2007), if we extrapolate our findings, the proportion of pregnant women with B12 levels below 150 pmol/L is likely to be much higher. More than a third of vegetarian/vegan women in our study had B12 deficiency, which was in keeping with studies from other UK adult population (Gilsing et al. 2010) and elsewhere (Pawlak et al. 2013). Thus these findings highlight the need for specific advice for the vegetarian/vegan population about potential sources of B12, as well as recommending them to have their B12 levels checked (and corrected) if they are planning pregnancy.

*Folate intake*

Our study shows that consumption of dietary folate has fallen by around 14% in the 10 years between the two surveys and nearly 50% of women are now consuming below the UK RNI. The Scientific Advisory Committee on Nutrition within the UK Food Standards Agency has recommended mandatory folic acid fortification of food products to the UK DOH (Scientific Advisory Committee on Nutrition 2006) which, if implemented, would increase folate intake and consequently serum levels in the population as it did in USA (Pfeiffer et al. 2007). However if mandatory fortification does occur, it is possible that improving folate levels can reduce B12 levels due to utilization of the available B12 (Selhub et al. 2009). In addition, B12 deficiency is the

strongest driver of homocysteine in a folate-replete population (Selhub et al. 2007b). Therefore, it is sensible to consider fortification of food products with B12 together with folate should there be a policy change in the UK or as a minimum, recommend B12 supplements in women in the periconceptional period especially if they are at high risk of B12 deficiency.

The strengths of our study are that this is the first study of its kind from the UK to evaluate B12 and folate among women in the pre-conceptional stage. Extensive data on these women including anthropometry, biochemical markers and dietary information allowed comparison of intake and serum levels adjusted for possible confounders. There were four important limitations of this dataset: a) limitations in sample size and potential selection bias; b) lack of sufficient biochemical data in the second survey; c) lack of data on other biomarkers such as holoTC or methylmalonic acid (MMA) and d) lack of haematological and clinical information relevant to the effects of B12. These will be discussed in turn.

Although the NDNS is meant to be a national survey representative of the UK population, inability to contact individuals, participant refusal to participate or obtain bloods and insufficient blood samples, information was only obtained from a third of the 3704 potentially eligible individuals in the first survey, from which the majority of our analysis is based on. From this, complete data was available from 299 women of child-bearing age who met our inclusion criteria so caution must be exercised when extrapolating the results to the wider UK population. A more up-to-date survey with a larger sample size of women sampled from populations representative of the whole of UK should be carried and their results considered prior to any national policy change with regards to B12 and folate intake recommendations.

Since we were able to compare the nutrient intakes between the two cohorts, which was predictive of serum levels in the earlier survey, we believe the that availability of serum B12 in the latter cohort may not necessarily change the findings. Hcy is a readily available marker in clinical practice as opposed to holoTC and MMA. Therefore, our findings are applicable to wider clinical practice. Any future

prospective studies involving preconceptional or pregnant women should include more clinical information as well as these biomarkers for a better evaluation of B12 status.

### 6.5. Conclusion

In conclusion, this study supports revision of the UK RNI to at least match the European recommendations and also calls for assessing maternal B12 status in pre-conception or early pregnancy.

# Chapter 7

# Incretin hormones in diabetes and potential role of GLP-1 receptor agonists in alleviating complications of hyperglycaemia

### 7.1. Incretin hormones

The incretin effect is described as the amplification of insulin secretion, which is observed when glucose is taken in orally as opposed to via an intravenous infusion to maintain the same plasma glucose concentrations (J.J. Holst and Gromada 2004). This potentiating effect of the incretin hormones accounts for around two-thirds of the insulin response after an oral glucose load. The 2 primary hormones responsible for this incretin effect are glucagon-like peptide 1 (GLP-1) and glucose-dependent insulotropic polypeptide (GIP).

GLP-1 is secreted by the enteroendocrine L-cells in the distal ileum and colon in response to a meal and its secretion is proportional to the energy intake of the meal (Field et al. 2010; C. Herrmann et al. 1995). It is formed from the cleavage of proglucagon, a hormone expressed in the pancreas, gastrointestinal (GI) tract and brain (Figure 7.1) (Seino et al. 2010). Tissue-specific cleavage of proglucagon occurs with glucagon being the predominant type produced in the pancreas and GLP-1, GLP-2 and oxyntomodulin produced in the GI tract and brain. Secretion of GLP-1 is stimulated directly by ingested gut contents coming into contact with receptors expressed on the surface of the L-cells as well as gut neuronal signals involving the vagus nerve (Rocca and Brubaker 1999). GLP-1 has a half life of around 2 minutes due to rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) and it is estimated that only 10-15% of newly secreted GLP-1 reaches the circulation due to metabolism by the gut and liver (Deacon et al. 1996).

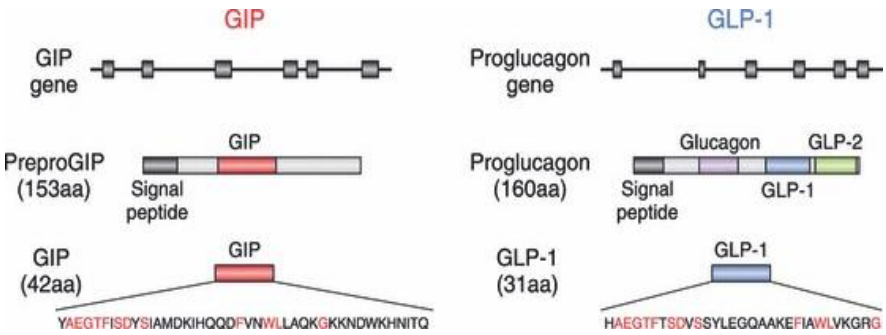


**Figure 7.1** Production of GIP and GLP-1 proteins**.** The glucose-dependent insulinotropic polypepide (GIP) gene is localized on human chromosome 17q21.3–q22 and comprises 6 exons. Proteolytic processing of preproGIP generates GIP that is secreted from K cells. The proglucagon gene is localized on human chromosome 2q36– q37 and comprises 6 exons. In the intestine, proteolytic processing of proglucagon generates glucagon-like peptide (GLP)-1 and GLP-2, whereas glucagon is produced in the pancreas. (from Seino et al. 2010)

GLP-1 has a multitude of effects on the body in addition to its primary function, which is glucose-dependent insulin secretion by the pancreatic beta-cells (Figure 7.2) (Baggio and Drucker 2007). These are not exhaustive but include inhibition of glucagon secretion by the alpha-cells, delayed gastric emptying and increasing satiety. The ability of GLP-1 agonists to cause sometimes dramatic weight loss has created plenty of interest its role as a regulator of energy balance. GLP-1 production has been discovered in the medulla and GLP-1 receptors in the hindbrain and hypothalamus (Goke et al. 1995; Merchenthaler et al. 1999). The hypothesis is that intraluminal GLP-1 secretion stimulates intestinal vagal nerve which provides positive feedback in hindbrain nuclei (central GLP-1 secretion may also contribute to this) (Barrera et al. 2011). These stimuli, in addition to those from gastric distension, are synthesised by the hindbrain which then produce efferent signals to reduced portion sizes.

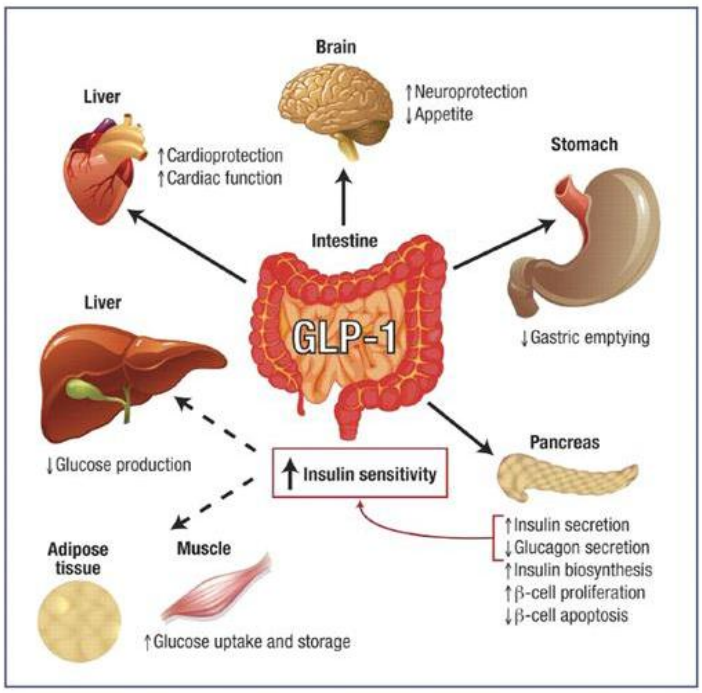


**Figure 7.2** GLP-1 actions in peripheral tissues. The majority of the effects of GLP-1 are mediated by direct interaction with GLP-1Rs on specific tissues. However, the actions of GLP-1 in liver, fat and muscle most likely occur through indirect mechanisms (from Baggio and Drucker 2007).

GIP is secreted by the K-cells of the upper small intestine and although some of its effects on the beta-cells of the pancreas are similar to GLP-1 (i.e. increasing insulin secretion and beta-cell proliferation), other effects it has on the body, particularly in the periphery are different. It causes increase in glucagon secretion by the pancreas, increase in fat accumulation in adipose tissue and bone formation (Seino et al. 2010). However, it does not reduce meal size similar to GLP-1, presumably because the location of GIP receptors in the brain are distinct from GLP-1 (Nyberg et al. 2005).

### 7.2. GLP-1 levels in type 2 Diabetes and insulin resistant states

*GLP-1 in type 2 diabetes*

In type 2 diabetes (T2D) patients, there only appears to be a slight decrease in postprandial GIP concentrations when compared to weight-, age- and sex-matched non-diabetic controls (M. B. Toft-Nielsen et al. 2001b) but the insulotropic effects of this hormone are significantly reduced or even lost (Krarup et al. 1987). The latter effects may be due to a decreased expression of the GIP receptor in the pancreatic beta-cells or defects in postreceptor mechanisms, particularly those involved with the second phase of insulin secretion (Vilsbøll et al. 2002).

However, the situation with GLP-1 seems to be somewhat reversed. Investigators who have done experiments by giving infusions of GLP-1, whilst maintaining a constant glucose by way of a hyperglycaemic clamp, have found that T2D patients clear the glucose load as efficiently as healthy controls, suggesting that the beta-cells are responsive to GLP-1 in T2D (Vilsbøll et al. 2002).

So if the insulotropic action of GLP-1 is maintained in T2D, is there submaximal release of GLP-1 to account for the impairment in incretin effect? The answer to this is less straightforward which may, in part, be due to heterogeneity in the studies conducted in terms of the glucose challenge given (e.g. glucose tolerance test (GTT), mixed meal tests) and assays used to measure GLP-1 (e.g. total GLP-1, intact GLP-1, etc.) (Calanna et al. 2013).

Some studies show that postprandial GLP-1 concentrations (as measured by total area under the curve (tAUC)) are significantly reduced by 20 – 50% in T2D as compared to controls (Elza Muscelli et al. 2008b; M. B. Toft-Nielsen et al. 2001b). However a recently conducted meta-analysis concluded that there was no difference in various measured of postprandial GLP-1 measures in T2D compared to controls (Calanna et al. 2013). Furthermore, subgroup analyses even showed increased GLP-1 responses in these patients during liquid mixed meal tests and 50g GTT and the authors suggested that the secretion pattern of GLP-1 may change in the course of the disease with hypersecretion early on followed by 'exhaustion' of the GLP-1 producing cells later on (Calanna et al. 2013). Indeed other studies have found that HbA1c and fasting plasma glucose (FPG) were independent negative predictors of GLP-1 secretion and the meta-analysis also corroborated this (Calanna et al. 2013; M. B. Toft-Nielsen et al. 2001b).

*GLP-1 in insulin resistance states*

Although the abovementioned studies demonstrated an association between deteriorating glycaemic control and a reduction in GLP-1 levels / its effect, there is another school of thought that the impairment of incretin effect is observed at the onset of insulin resistance too. In an experiment where insulin resistance was induced in healthy individuals by 5 days of dexamethasone treatment, the investigators found

that the subjects who developed insulin resistance showed a reduction in the incretin effect from 71% to 58% (a change of 18%) before and after the treatment during a 75g GTT/isoglycaemic intravenous glucose test (Jensen et al. 2010). Additionally, those who developed impaired glucose tolerance as well demonstrated a reduction in their incretin effect from 67% to 32% (a change of 52%) suggesting that there is an impairment in β-cell response to incretins early on but its secretory capacity does not decrease till later (Holst et al. 2011; Jensen et al. 2010).

These observations may, of course, be due to obesity which has been shown to be an independent negative predictor of GLP-1 response in several studies (Elza Muscelli et al. 2008b; M. B. Toft-Nielsen et al. 2001b). Even among subjects with NGT, obese individuals had significantly lower incremental AUC for total GLP-1 compared to lean individuals during GTT (Knop FK 2008). The former group in this study also demonstrated a reduced incretin effect although they did not differ from lean individuals in terms of fasting plasma glucose, post-GTT glucose excursion or HbA1c once again suggesting that impairment of the incretin effect may be a very early pathophysiological trait before the development of impaired glucose tolerance (IGT) or T2D (Knop FK 2008).

### 7.3. GLP-1 levels in gestational diabetes

From the above, it seems to be evident that there is an impairment of the incretin effect in insulin resistance states, particularly when accompanied by obesity. So it is probable that a similar effect will be observed in pregnancy, which is a physiological state of insulin resistance.

A search on PubMed found 6 articles which measured GLP-1 levels or the incretin response in pregnancy (Bonde et al. 2013; Cypryk et al. 2007; Gonzalez et al. 2011; Kosinski et al. 2013; Lencioni et al. 2011; Reyes-Lopez et al. 2014). Table 7.1 shows a summary of these studies. It is difficult to draw any definite conclusions from these studies due to the wide heterogeneity in gestation of GLP-1 measurement, the glucose challenge used and type of GLP-1 measurement done (e.g. mean GLP-1, AUC GLP-1, total GLP-1 all measured in different studies). These will be discussed in more detail in Chapter 8.

**Table 7.1** Summary of studies which have measured glucagon-like peptide-1 (GLP-1) levels or incretin response in gestational diabetes and normal glucose tolerance pregnancies

| Reference | Participants (n) | Stage of pregnancy | Glucose challenge | Components of GLP-1 response analysed (analytical method) | Key findings |
|---|---|---|---|---|---|
| Bonde et al. 2013 | GDM (11)<br>NGT (8) | Third trimester (TT) &<br>3-4 months post-partum (PP) | 4-hour liquid meal | Total AUC, fasting GLP-1 (RIA) | - GDM vs. NGT in TT: non-significant ↓ total AUC GLP-1 (5.5 vs.7.3 nM x min)<br>- GDM at TT vs. PP: ↓ total AUC GLP-1 (5.5 vs. 8.4 nM x min, p=0.005)<br>- GDM vs. NGT in TT and PP: ↓ fasting GLP-1 levels at both time-points (p=0.03 and 0.04)<br>-GDM at TT and PP vs. NGT: ↑ GIP levels |
| Lencioni et al. 2011 | GDM (12)<br>NGT (16) | TT (mean 27 weeks) & 12-18 months PP | 3-hour 100g GTT | Mean GLP-1, total AUC (RIA kit for active GLP-1) | - GDM vs. NGT at TT: no difference in GLP-1 AUC; ↓ mean GLP-1/mean glycaemia ratio<br>-GDM vs. NGT at PP: ↓ GLP-1 AUC (2542 vs. 10092, p<0.05) |
| Cypryk et al. 2007 | GDM (13)<br>NGT (13) | TT (mean 27-29 weeks) | 75g GTT | Active GLP-1 and total AUC (RIA) | - GDM vs. NGT: ↑ fasting GLP-1 levels; no difference in total AUC<br>- GDM vs. NGT: positive correlations between fasting GLP-1 and insulin concentration and resistance (r=0.54, p=0.004 and r=0.43, p=0.029) |
| Kosinski et al. 2013 | GDM (10)<br>NGT (8) | TT &<br>3-4 months PP | 4-hour 50g GTT and IV glucose | No GLP-1 levels measured. Incretin effect for AUC insulin, GIGD (N/A) | - GDM at TT vs. PP: ↑ incretin effect at PP (31 vs. 56%, p=0.02); GIGD reduced to diabetic levels in GDM but ↑ PP (37% vs. 58%, p=0.03); no difference in NGT |
| Reyez-Lopez et al. 2014 | GDM (90)<br>NGT (108) | TT | Fasting bloods | Mean GLP-1 (RIA) | - GDM vs. NGT: ↑ fasting GLP-1 levels (p=0.004) |
| Gonzalez et al. 2001 | GDM (15)<br>NGT (22) | Delivery | Random bloods at C-section | Active GLP-1 (ELISA) | -GDM vs. NGT: No difference in GLP-1 levels; positive correlation between GLP-1and HOMA-IR |

GDM: gestational diabetes mellitus, NGT: normal glucose tolerance, TT: third trimester, PP: post-partum, AUC: area under the curve, GTT: glucose tolerance test, IV: intravenous, GIGD: gastrointestinally mediated glucose disposal, RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbant assay, HOMA-IR: homeostasis model assessment of insulin resistance

## 7.4. GLP-1 and GLP-1 receptor agonists in the treatment of diabetes and associated complications

### 7.4.1. Clinical use of GLP-1 receptor agonists

The first GLP-1 receptor agonist (GLP-1 RA) developed and approved for clinical use was exenatide, which is synthetically prepared from exendin-4, an incretin mimetic derived from the saliva of the Gila monster (Eng et al. 1992). It is 53% homologous to native GLP-1 and is resistant to degradation by DPP-4 due to an amino acid substitution. Liraglutide, the second GLP-1 RA to come into clinical practice has 97% homology to native GLP-1 but has a longer half-life than Exenatide due to its resistance to DPP-4 inhibition being mediated by binding to serum albumin (Tahrani et al. 2016). Due to this, it can be administered once a day via a subcutaneous injection of 0.6, 1.2 or 1.8mg.

Multi-national randomised control trials, head-to-head comparisons with other GLP-1 RA's and meta-analyses have shown remarkable benefits of Liraglutide in terms of glycaemic control and weight loss, amongst other benefits. Mean haemoglobin A1c (HbA1c) decrease is 1.27% (95% CI 1.13 -1.41) after 26 weeks treatment and mean fasting a postprandial blood glucose decrease 1.82 mmol/l (1.57 – 2.07) and 1.7 – 2.1 mmol/l (monotherapy only) (Tahrani et al. 2016). Mean weight loss on liraglutide 1.8mg was 1.51 (2.67 – 0.31) kg (Potts et al. 2015).

Three further GLP-1 RA's have recently become available (Lixisenatide, Albiglutide and Dulaglutide) which vary slightly in their composition and duration of action. Long-term cardiovascular outcome data are eagerly awaited on these drugs.

### 7.4.2. Non-glycaemic effects of GLP-1 receptor agonists

While the GLP-1 RA class of drugs initially garnered a lot of attention for their dramatic effects on glycaemic control and weight loss, more recent attention has also been on the cardiovascular protection it confers. This might be an indirect effect of its glucose- and weight-lowering effect but recent body of evidence suggests that it might have a direct effect on the endothelium too.

Clinical studies have shown that in patients with ischaemic heart disease and heart failure, the infusion of GLP-1 improved left ventricular function, myocardial oxygen

consumption and quality of life, an observation which was noticed in diabetics and non-diabetics (Nikolaidis et al. 2004; Sokos et al. 2006). Meta-analysis data is available supporting the benefit of Liraglutide and other GLP-1 RAs in lowering systolic blood pressure (-4.2mmHg compared to insulin) and total cholesterol, LDL and triglycerides but there is no benefit on HDL (Katout et al. 2014; F. Sun et al. 2015a; F. Sun et al. 2015b). There is also good evidence that GLP-1 improves endothelial dysfunction, as measured by flow-mediated dilatation of the brachial artery in diabetic patients (Nystrom et al. 2004). I will discuss these observations in more detail, together with possible mechanisms below and in Chapter 9.

## 7.5. GLP-1 receptor agonists in the alleviation of hyperglycaemia-oxidative stress in endothelial cells: focus on molecular mechanisms

### 7.5.1. Endothelial dysfunction

Vascular disease is central to the pathogenesis of several complications of diabetes mellitus (DM); small vessel disease is responsible for retinopathy, nephropathy and neuropathy while atherosclerosis in large vessels accounts for coronary, carotid and peripheral vascular disease (Schalkwijk and Stehouwer 2005). Macrovascular disease is responsible for 50 – 75% of mortality in DM, mainly due to ischaemic heart disease and cerebrovascular disease. The clinical features of vascular involvement in diabetic patients and the key signaling insults implicated in them are summarised in Figure 7.3 (Kolluru et al. 2012).

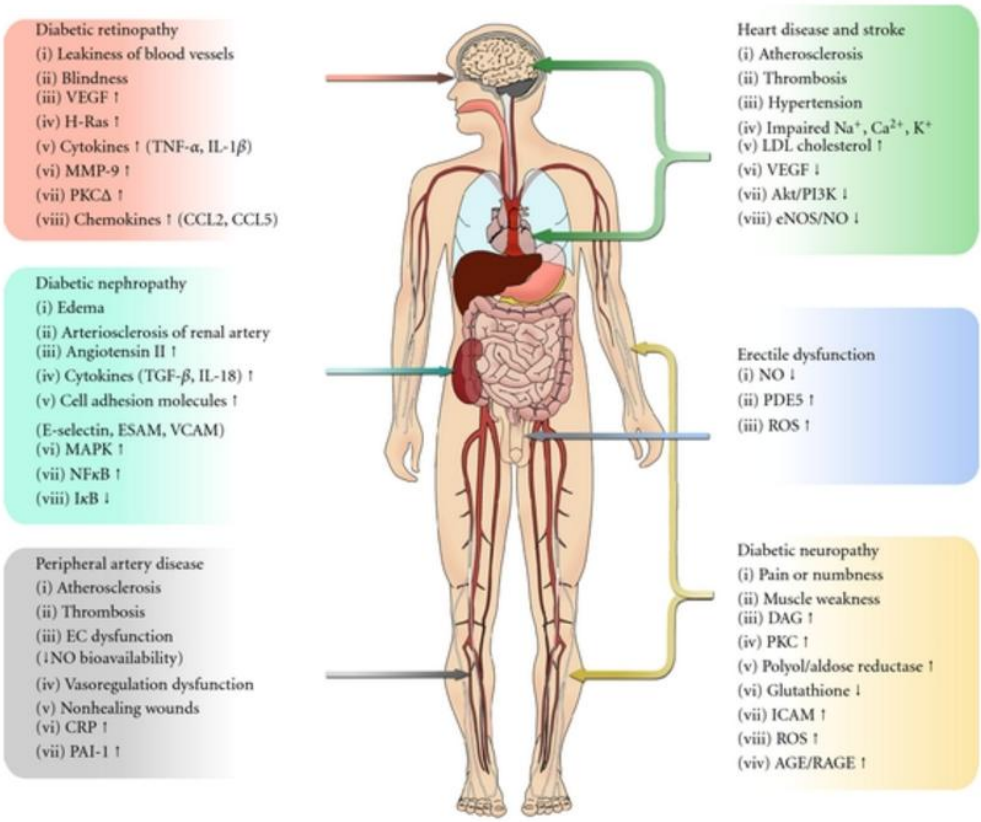


**Figure 7.3** Diabetic vascular disease effects and symptoms. Various pathophysiological conditions affected in the body due to diabetic vascular disease are illustrated. Prominent symptoms of diabetes mediated abnormalities are indicated for each condition. (See text for details on selected pathways)

The vascular endothelium is a layer of biologically active cells lining the inner wall of blood vessels and plays an important role in the regulation of vascular tone, hemostasis and inflammation (Schalkwijk and Stehouwer 2005). The tonicity of the endothelium is a balance between vasoconstricting factors (such as endothelin-1, prostaglandin H2, reactive oxygen species (ROS) and angiotensin 2) and vasorelaxing factors, the main one being endothelial nitric oxide (NO) (Dai et al. 2013). NO, which is produced by endothelial nitric oxide synthase (eNOS), has several protective functions in endothelial cells, conferring protection against atherosclerosis and plaque build-up (reviewed in *Kim et al*) (J. A. Kim et al. 2006). These include inhibiting platelet adhesion and aggregation, decreasing the expression of proteins involved in atherogenesis (e.g.VCAM-1, ICAM-1, MCP-1), reducing vascular permeability, inhibiting the oxidation of low density lipoprotein to its proatherogenic form and scavenging superoxide (X. L. Du et al. 2001).

Nitric oxide synthase (NOS) exists in 3 isoforms in endothelial cells: neuronal NOS (nNOS), inducible NOS (iNOS) and eNOS. Some factors which stimulate eNOS production are sheer stress due to increased endothelial blood flow and the phosphatidyl-inositol-3 kinase (PI3K)-Akt pathway by insulin and vascular endothelial growth factor (VEGF) (Fulton et al. 1999). Activation of the PI3K-Akt system phosphorylates eNOS at Ser(1177). 5'-adenosine monophosphate activated protein kinase (AMPK) is also shown to phosphorylate eNOS at more than one site (discussed in detail below).

Endothelial dysfunction is defined as a diseased state in the endothelium whereby it transforms into a vasoconstrictor, proinflammatory and prothrombotic state (G. Yang et al. 2010). Importantly, it has been shown that endothelial dysfunction is associated with the release of pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), which is an early initiating factor of atherosclerosis in DM (Dai et al. 2013). One of the main insults in endothelial dysfunction is a decrease in NO-mediated vasorelaxation, which can be due to reduced eNOS phosphorylation/activation, eNOS uncoupling (i.e. greater production of monomerised eNOS which generates superoxide anions instead of NO that is produced by the homodimer eNOS), increased arginase (which cleaves L-arginine, the precursor of NO) and reduced tetrahydrobiopterin (BH4, a compound which stabilises eNOS in its active homodimer form) [reviewed in *Kolluru et al*] (Kolluru et al. 2012).

The signaling mechanisms implicated in the development of endothelial dysfunction due to hyperglycaemia are discussed in more detail in the next section.

### 7.5.2. Generation of oxidative stress in endothelial cells

Oxidative stress, which occurs when the rate of production of ROS exceeds their scavenging, has been linked to many of the vascular complications of DM, including

endothelial dysfunction (King and Loeken 2004). On a tissue level, ROS has been shown to affect blood flow in the retina and reduce conduction in peripheral nerves (Hounsom et al. 2001; Kowluru and Kennedy 2001; Kunisaki et al. 1995).

The endothelium is particularly sensitive to the damaging effects of ROS due to its negative effects on NO synthesis among other factors (Son 2007). There are three main sources of ROS production in vascular cells, namely (1) xanthine oxidase, (2) NADH/NADPH oxidase (i.e. nicotinamide adenine dinucleotide phosphate hydrogen) and (3) eNOS (reviewed in *Cai and Harrison*) (Cai and Harrison 2000). The most toxic of ROS are the superoxide anion ($O2^-$), hydroxide peroxide ($H_2O_2$) and additionally $O2^-$ can combine with nitric oxide to produce peroxynitrite ($ONOO^-$). Superoxide dismutase (SOD), catalase and glutathione peroxidase are protective enzymes that break down the ROS. The formation and clearance of ROS are summarised in Figure 7.4 (Droge 2002).

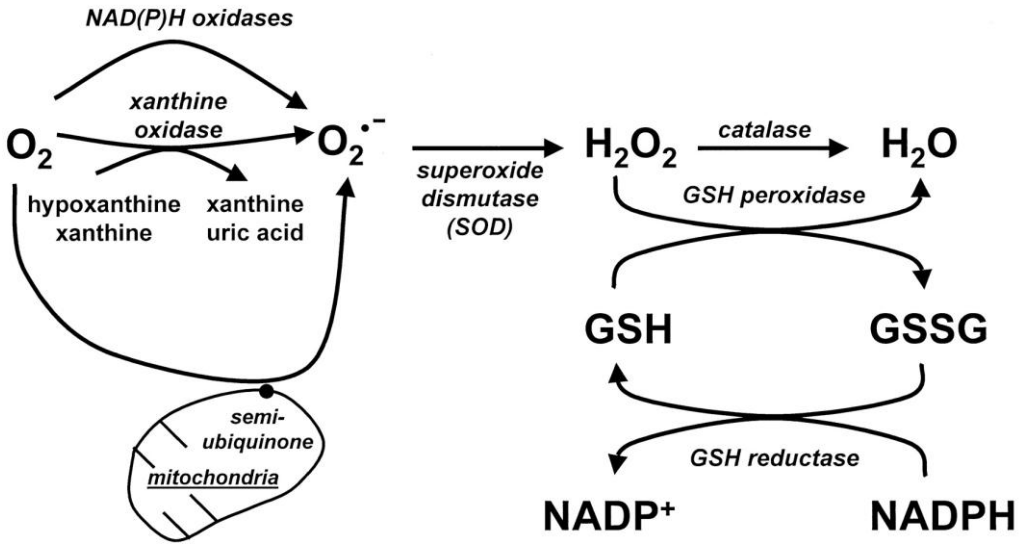


**Figure 7.4** Pathways of reactive oxygen species (ROS) production and clearance. GSH: glutathione, GSSG: glutathione disulfide (from Droge 2002)

Chronic hyperglycaemia in the context of DM causes increased oxidative stress in several tissues due to excess production of ROS and impaired clearance (Nishikawa et al. 2000). Figure 7.5 shows how increased intracellular glucose increases ROS production and inhibits its clearance through several interacting pathways (King and Loeken 2004; Schalkwijk and Stehouwer 2005). In summary, the 4 main pathways

activated are:

1) Polyol pathway (causing increased production of sorbitol by aldose reductase)
2) Hexosamine pathway (i.e. production of glucosamine-6-phosphate from fructose-6-photose)
3) Protein kinase C (PKC) pathway
4) Formation of advanced glycation end-products (AGE) by non-enzymatic reactions

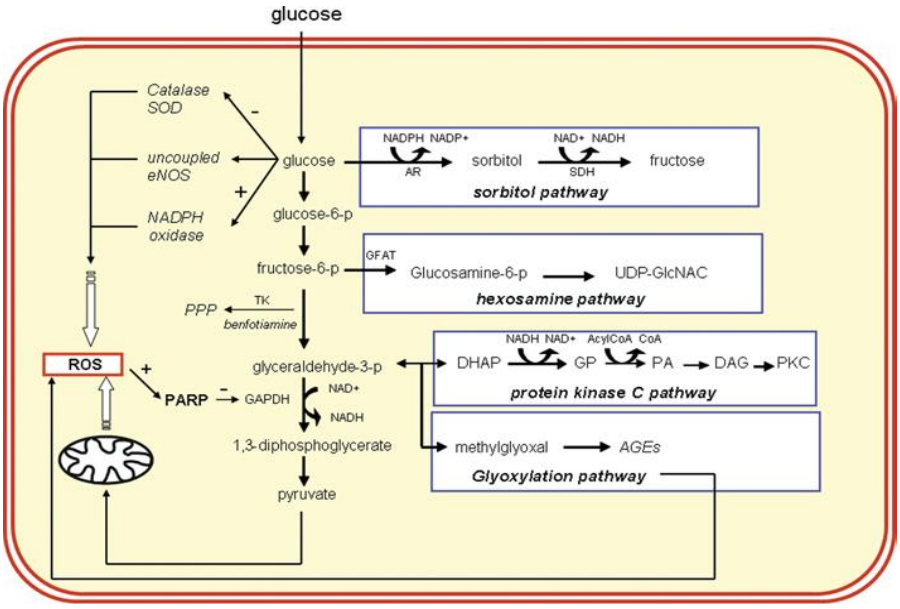


**Figure 7.5** Potential mechanisms by which hyperglycaemia and its immediate biochemical sequelae induce hyperglycaemic damage (See text for details on selected pathways) (from Schalkwijk and Stehouwer 2005)

PKC is a stimulator of NADPH oxidase, which is responsible for the oxidation of NADPH to $NADP^+$. The resulting reduced NADPH/ $NADP^+$ ratio contributes to the generation of ROS by (1) reducing the regeneration of an antioxidant glutathione (GSH) from oxidised glutathione (GSSG) and (2) decreasing the activity of catalase, the enzyme which converts $H_2O_2$ to $H_2O$. The generation of PKC and its toxic effects on cells are summarised in Figure 7.6 (King and Loeken 2004). These processes affect vascular tissue as well because glutathione scavenging and NADPH levels have been shown to be reduced in endothelial cells exposed to high glucose (Kashiwagi et al. 1996).

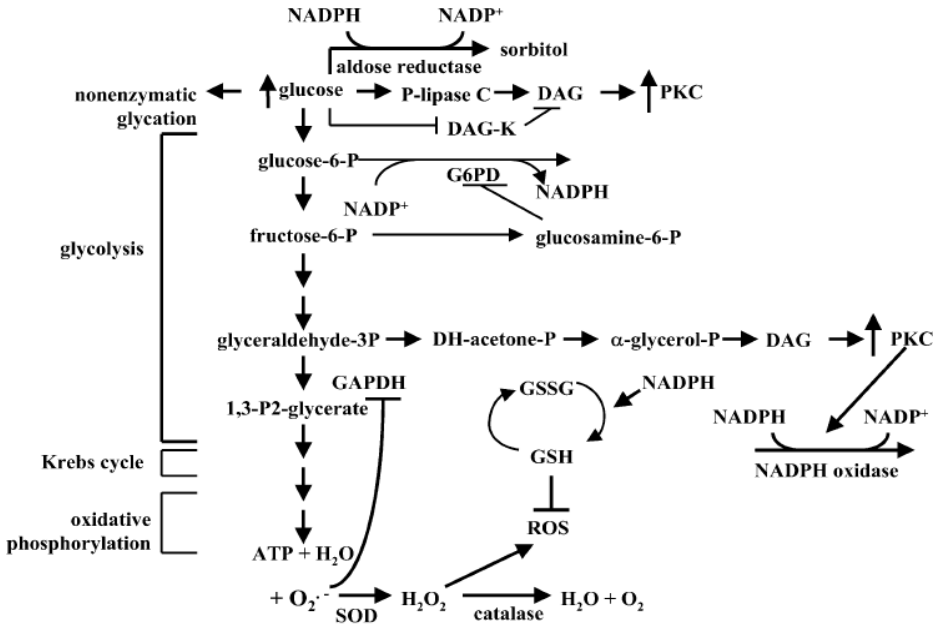


**Figure 7.6** Schematic diagram of pathways that contribute to oxidative stress in response to increased glucose flux (See text for details of selected pathways) (from King and Loeken 2004)

### 7.5.3. Consequences of oxidative stress in the vascular endothelium: cellular damage and apoptosis

Oxidative stress leads to cellular death and damage directly by causing DNA strand breaks and cell death (X. Du et al. 2003) or indirectly by altering expression of proapoptotic and antiapoptotic genes. Some of the genes which are activated by oxidative stress are those involved in the p38/mitogen activated protein kinase pathway, caspase and poly-ADP-ribosyltransferase which lead to cell death (reviewed in *King et al*). Oxidative stress also causes altered DNA biding of transcription factors such as nuclear factor kappa beta (NF-κB) and p53, which are responsible for the expression of several proapoptotic genes (Marshall et al. 2000; B. Schisano et al. 2011).

Peroxynitirite ($ONOO^-$), another ROS which is generated by iNOS acting on endothelial macrophages and smooth muscle cells, further contributes to cellular damage by increasing lipid peroxidation and oxidation of LDL (Griendling and FitzGerald 2003).

*Hou et al* studied the mechanism of ROS-induced apoptosis in endothelial cells and found upregulation of Nox 4, an isoform of NADPH oxidase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression/phosphorylation and caspase-3 proteins in a concentration-dependent manner when exposed to hyperglycaemia (Figure 7.7) (Hou et al. 2015). Further experiments with silencing of Nox4 and PTEN genes showed that activation of PTEN by ROS led to apoptosis by decreasing intergrin-like kinase (ILK) activity, Akt phosphorylation and increasing Bcl-2 associated X protein (BAX) expression and caspase activation. However, this study showed that PTEN overexpression and caspase-3 activation are the main, but not only molecules upregulated by Nox4-induced ROS suggesting that other signaling pathways mediating cell damage/apoptosis need to be elucidated.

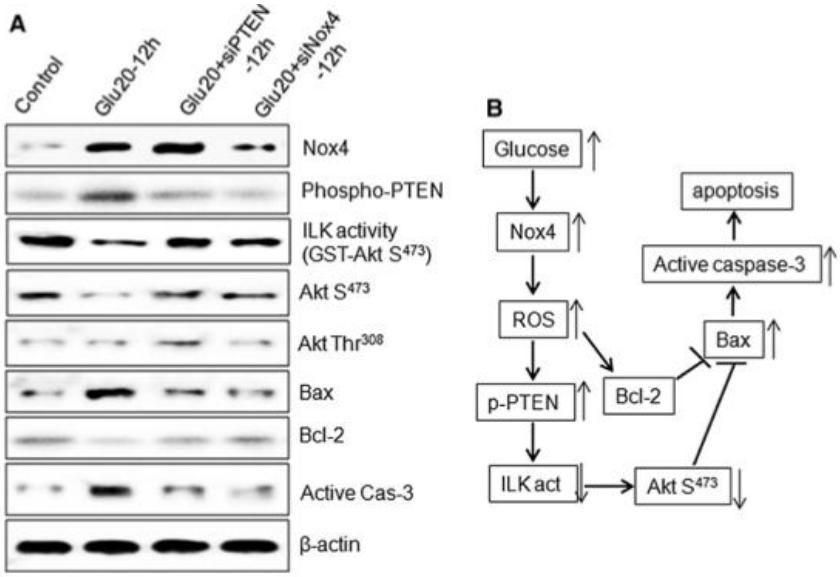


**Figure 7.7** Signaling pathways activated by glucose. **A)** Representative Western blots. Glu20: 20 mM glucose. siPTEN: PTEN siRNA transfection; siNox4: Nox4 siRNA transfection. **B)** Diagram of high glucose activated signaling. High glucose increased Nox4 expression and reactive oxygen species production. Reactive oxygen species increased PTEN phosphorylation and decreased Bcl-2 expression. PTEN- inhibited ILK activity and Akt S473 phosphorylation. The decreases in Bcl-2 expression and Akt phosphorylation lead to an increase in Bax expression and activation of caspase-3 (from Hou et al, 2015)

Oxidative stress is only one of the mechanisms by which hyperglycaemia leads to endothelial dysfunction. Other possibilities include direct inhibition of the PI3K/phospho-AKT/eNOS pathway (discussed below).

### 7.5.4. PI3K/phospho-Akt signaling pathway

Activation of receptor tyrosine kinases stimulate PI3K either directly or through phosphorylation of intermediate receptors, such as insulin receptor substrate (IRS). PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), and the latter directly phosphorylates Akt at Ser(473) and Thr(308), by co-binding with PDK-1 (Manning and Cantley 2007). The PIP2 to PIP3 conversion can be reversed by the PIP3 phosphatase PTEN. Additionally, PIP3 indirectly phosphorylates Akt at Ser(473) alone by activating ILK.

The PI3K-Akt pathway which is stimulated by insulin, via the insulin receptor substrate, plays an essential role in controlling the pleiotropic effects of insulin on vascular function (Song et al. 2007). Some of the important downstream effects of Akt (and its active phosphorylated form phospho-Akt) on the cells are increasing glucose uptake by GLUT-4 translocation and activating mouse double minute 2 homolog (MDM2) (which is the main negative regulator of p53, an important pro-apoptotic factor (B. Schisano et al. 2011; Q. Wang et al. 1999)). In relation to protecting endothelial cell function, Akt is a powerful activator of eNOS synthesis (Dimmeler et al. 1999) and in pathological states such as DM, the eNOS can become uncoupled and contribute to formation of reactive nitrogen species (RNS) (Kolluru et al. 2012). Figure 7.8 summarises some of the other mechanisms by which of that Akt promotes cell survival and proliferation (Manning and Cantley 2007).

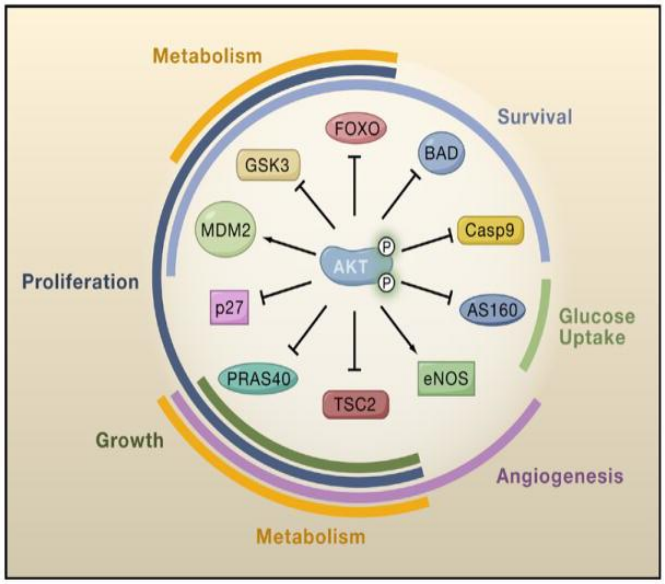


**Figure 7.8** Cellular Functions of Akt Substrates. Akt-mediated phosphorylation of these proteins leads to their activation (arrows) or inhibition (blocking arrows). Regulation of these substrates by Akt contributes to activation of the various cellular processes shown (i.e., survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis). As illustrated by these ten targets, a high degree of functional versatility and overlap exists amongst Akt substrates (see text for details of selected pathways) (from Manning and Cantley 2007).

The generation of ROS contributes to insulin resistance by inhibiting activation of IRS by insulin and consequently there is less Akt phosphorylation/activation. Since Akt is an important negative mediator of forkhead box class O (FOXO) and the cell damage associated with this, one can understand how hyperglycaemia can worsen the damage caused by ROS by reducing Akt activation (Ponugoti et al. 2012). The main negative regulator of Akt is PTEN and a study has shown that hyperglycaemia causes Akt inhibition by liver kinase B1 (LKB1) dependent PTEN activation which is mediated by peroxynitrite, a type of RNS (Song et al. 2007).

### 7.5.5. Phospho-AMPK signaling pathway

AMPK is activated in situations of increased adenosine monophosphate : adenosine triphosphate (AMP:ATP) ratio (e.g. hypoxia, glucose deprivation). It serves to inhibit anabolic processes which consume ATP (e.g. fatty acid and protein synthesis) and stimulate catabolic ones (e.g. glucose uptake, fatty acid oxidation) (Shirwany and Zou 2010). Other factors which lead to AMPK stimulation are cytokines (e.g. leptin, adiponectin) and drugs such as metformin, thiazolidinediones (Batchuluun et al. 2014; Shirwany and Zou 2010). Activation of AMPK is dependent on its phosphorylation by either LKB1 (a serine/threonine tumour suppressor kinase) or CaMKK-β (calcium/calmodulin-dependent protein kinase kinase-beta). However, the

downstream targets of AMPK are diverse and yet to be completely elucidated. In summary, they include stimulation of lipid metabolism (by downregulating acetyl-CoA carboxylase) and influencing other cell signaling (e.g. eNOS and IRS-1) (Z. P. Chen et al. 1999; Jakobsen et al. 2001).

There is convincing evidence that AMPK has beneficial effects on the vascular endothelium, which is mediated in part by the phosphorylation of eNOS on more than one site, namely Ser(1177) and Ser(633) (Z. P. Chen et al. 1999; Z. Chen et al. 2009). Such AMPK-stimulated eNOS phosphorylation occurs following stimulation by VEGF, peroxisome proliferator-activated gamma agonists (e.g. thiazolidinediones), AICAR and metformin. Of these, the best studied pathway is AMPK activation by metformin, which is mainly PI3K-dependent, although NO may also directly stimulate AMPK (Zhang et al. 2008; Zou et al. 2004).

Aside from its effect on eNOS, AMPK has been shown to exert a protective effect in the endothelium by inhibiting the production of ROS by NAPDH oxidase and reducing activation or signaling by c-JUN N-terminal kinases (JNK), NF-**κ**B and VCAM-1 following exposure to oxidants such as $H_2O_2$ or TNF-**α** (Cacicedo et al. 2004; Dixit et al. 2008; Fisslthaler and Fleming 2009). Additionally, silencing of AMPK decreases expression of antioxidants such as SOD and catalase (Colombo and Moncada 2009).

Some of the processes associated with endothelial cell apoptosis due to high glucose exposure, such as inhibition of fatty acid oxidation, increase in diacylglycerol (DAG) synthesis (the precursor of PKC) and reduced insulin mediated phosphorylation of Akt were all reduced in the presence of an AMPK activator (Ido et al. 2002). In the same experiment, AMPK activation prevented caspase-3 activity, providing further evidence of its protective role in hyperglycaemia.

### 7.5.6. Anti-inflammatory effects of GLP-1

From the clinical studies quoted in Section 7.4.2, it is apparent that the cardiovascular benefit of GLP-1 RAs are partly due to direct effects on the myocardium and endocardium. Indeed, GLP-1 receptors have been found in both animal and human myocardia as well as in parts of the brain involved in autonomic function, such as the hindbrain and hypothalamus (Bullock et al. 1996; Wei and Mojsov 1995). Some of the cardiovascular benefits of direct GLP-1 activation of the hearts of animal models include reduction in left ventricular (LV) end-diastolic pressure, improved LV contractility and improved myocardial reperfusion following ischaemia (Gros et al. 2003; Matsubara et al. 2011). The myocardial protection provided by GLP-1 is in part an anti-apoptotic effect mediated through the activation of cAMP and PI3K via its binding to the GLP-1 receptor (Buteau et al. 1999). This observation suggests that Akt may be an important downstream target of the GLP-1 signaling pathway. However, a GLP-1 receptor independent pathway has also been proposed for improved left ventricular function after ischaemic injury by causing vasodilatation due to NO release (Ban et al. 2008). Animal studies support a role for GLP-1 acting on GLP-1 receptors to cause renal artery relaxation and reduce systolic blood pressure in hypertensive rats, which was mediated by the protein kinase A/AMPK/NO pathway (Liu et al. 2012).

### 7.5.7. Amelioration of endothelial dysfunction by GLP-1 / GLP-1 receptor agonists

Endothelial cells have also been shown to possess GLP-1 receptors, which are stimulated by agonists such as Liraglutide (Dai et al. 2013; Ishibashi et al. 2010).

The potential mechanisms by which GLP-1 acts on endothelial cells is summarised in Figure 7.9 (Saad et al. 2015). Most of the studies investigating the action of GLP-1 RA on endothelial cells have attributed its benefits to enhanced eNOS production and consequent NO-mediated vasodilatation and anti-inflammatory effects. However, other mechanisms such as inhibition of endothelin-1 (a potent vasoconstrictor) and decreased cell apoptosis may also be present (Dai et al. 2013; Saad et al. 2015).

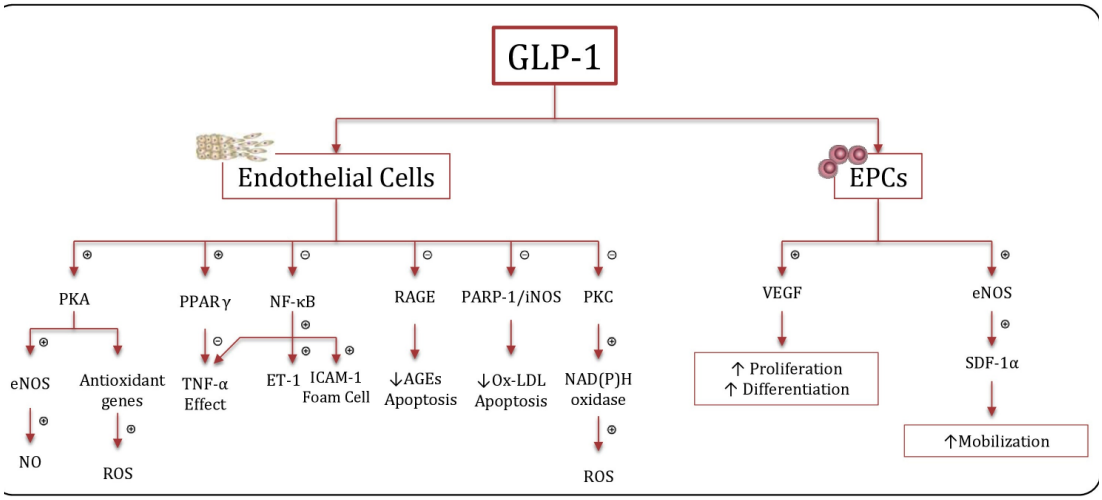


**Figure 7.9** Biochemical mechanisms by which GLP-1exerts its protective effect in endothelial cells and erythrocyte progenitor cells (EPCs). The '+' signs indicate the pathways that GLP-1 activates and the '-' signs ones that it inhibits (see text for details of selective pathways) (from Saad et al 2015).

Initial studies investigating the benefit of GLP-1 RAs on endothelial function showed that their antioxidant and anti-inflammatory effects were mediated by inhibition of PKC- **α** translocation to the cell membrane, NADPH-oxidase and NF-**κ**B (Shiraki et al. 2012). However in this study, oxidative stress was induced by TNF-α so although it provides some insight into the downstream effects of GLP-1 RA's it cannot be ascertained if the same mechanisms are at play in a hyperglycaemia or diabetic milieu.

### 7.6. Next steps

I will now show, via a prospective case-control study, how GLP-1 levels may be used as a biomarker in diagnosing and risk stratifying GDM pregnancies (Chapter 8) and in a laboratory project on endothelial cells, possible mechanisms by which liraglutide can ameliorate endothelial dysfunction in a hyperglycaemic environment (Chapter 9).

# Chapter 8

# GLP-1 profile during glucose tolerance test in gestational diabetes: a prospective case-control study

## 8.1. Hypothesis and aims

The hypothesis of this clinical study is that impairment of the GLP-1 response is present in women diagnosed with gestational diabetes mellitus (GDM) compared to those with normal glucose tolerance (NGT) and that this is particularly associated with hyperglycaemia following a glucose tolerance test (GTT).

The primary aim of the study was to determine a difference in GLP-1 response (as measured by total area under the curve) between GDM and NGT women during a 2–hour 75g GTT.

Secondary aims were to examine the GLP-1 profile by half-hourly sampling during the GTT, specifically for any differences between GDM cases and controls and determine if GLP-1 levels were predictive of high glucose levels 2-hours after a oral GTT.

## 8.2. Methods

### 8.2.1. Study design and justification

A prospective case-control study of pregnant women was conducted with the participants recruited during their glucose tolerance test (known as the 'PRiDE-GLP-1' study).

Such a prospective design was thought to be the best study method to answer the research questions because, at the time of the GTT and sampling of GLP-1 levels, neither the patient nor the research team knew whether she was going to test positive for GDM thereby minimising selection bias. Since the primary aim of the study was to detect differences in GLP-1 levels between GDM and normal glucose tolerance (NGT) women, recruitment of women at the time of their GTT was the optimal time point to identify GDM cases and controls. The National Institute for Health and Care Excellence (NICE) 2015 selective screening criteria was used to screen high-risk pregnant women with a GTT (see Section 8.2.2), which also would minimise exposure bias because both cases and controls had similar risk factors for the development of GDM.

### 8.2.2. Participant selection

The pregnant women for this study were recruited from the larger cohort of the 'Micronutrients in pregnancy as a risk factor for gestational diabetes and effects on mother and baby' (PRiDE Study) cohort. The primary research question of the PRiDE study is, "what is the role of B12, folate and homocysteine on the risk of GDM and markers of metabolic disease in the offspring?". It is a prospective longitudinal cohort in early pregnancy (n=4500) of two ethnic groups who are at high metabolic risk and are eligible to undergo a GTT at 24-28 weeks gestation. Detailed pheno- and genotyping of mothers (history, anthropometry, psychological well-being, blood and urine sampling) and neonates (anthropometry, cord blood, cord and placenta) is being done. The advantage for conducting my study on the cohort of women already enrolled in PRiDE is that these women and their infants are very well characterised throughout pregnancy, which will enable me to study several secondary outcomes that are potentially relevant to GLP-1 profile and glycaemia in pregnancy.

I conducted the PRiDE GLP-1 study over a 2-year period (October 2014 to September 2016) in 2 NHS Trusts in the West Midlands (George Eliot Hospitals NHS Trust and University Hospitals of Coventry and Warwickshire). The Participant Information Sheet and Consent form are provided in Appendix 3.1-3.2. The inclusion criteria for selection of participants for this study are:-

- Pregnant women between 15 and 30 weeks gestation
- Age 18 – 45 years
- High-risk group for GDM (at least 1 of the following risk factors): BMI >30 $kg/m^2$; previous GDM; previous unexplained still birth or baby >4.5kg; first degree relative with diabetes, ethnic minority group (South Asians, Middle-Eastern, Afro-Caribbean), age >35 years

The abovementioned definition of high-risk group for GDM is derived from the NICE 2015 guidelines and is used by several NHS Trusts in the country (National Institute for Health and Care Excellence 2015a) . According to a report from the Office of National Statistics in 2014, the proportion of older women having babies has been steadily rising in the last 4 decades, which was the reason for expanding the inclusion criteria to include pregnant women up to 45 years (Office for National Statistics

2015). Since older age is also an independent risk factor for GDM, this age range would additionally identify women with the highest risk of the condition.

Exclusion criteria are as follows

- Pre-gestational diabetes mellitus (Type 1 or 2)
- Multiple pregnancies
- Withdrawal of consent from the parent PRiDE study during the GTT

Pregnant women meeting the eligibility criteria were approached for the study when they attended for their GTT. I explained the study to them in person and provided the participant information sheet. Informed written consent was obtained before proceeding with the blood collection.

The timing of the GTT, and thereby recruitment into the PRiDE GLP-1 study, was in accordance with the NICE 2015 guidelines. It recommends that pregnant women meeting the selective screening criteria be offered a 75g oral GTT at 24-28 weeks gestation except if the women had a diagnosis of GDM in a previous pregnancy as a risk factor. In that subgroup of patients, the guidelines state recommend either self-monitoring of capillary blood glucose from early pregnancy or an 'early GTT' soon after booking. Therefore some women in my cohort were recruited during their 'early GTT' but if that was normal, the women were still offered a second GTT at the later gestation.

### 8.2.3. Blood sampling protocol

The GTT protocol involves women attending the maternity department in the morning after fasting for at least 10 hours the previous night (plain water only is permitted). A fasting blood test for serum glucose was obtained followed by the consumption of 200ml of Procal (containing 75g of anhydrous glucose) over a maximum of 2 minutes. The women were then asked to sit in the waiting room of the antenatal clinic before another serum sample for glucose was taken at 2 hours.

For the women who had consented to take part in the GLP-1 study, a 22G or 24G cannula was inserted in the fasting state. Blood samples for plasma (to measure GLP-1 and other gut hormones, such as GIP and glucagon) and serum (to measure glucose and insulin) were drawn from this intravenous cannula a t=0, 30, 60, 90 and 120 minutes of the GTT. At each time point, around 5mls of blood was obtained which was divided equally into 1 plasma and 1 serum tube. The tubes were centrifuged at 2000 rpm for 15 minutes at room temperature within 5 minutes of collection and separated into 2 aliquots each of plasma and serum. These aliquots were kept in a 4$^{o}$C fridge until the end of the GTT, when they were transferred to a -80$^{o}$C freezer for storage until analysis.

Due to the short half-life of plasma GLP-1 ($T_{1/2}$ 4 – 24 hours in a ethylene diamine tetra-acetic (EDTA) tube at room temperature) (Adaikalakoteswari et al. 2015b), 40ul of a DPP-4 inhibitor, Diprotin A (Enzo LifeSciences, catalogue number ALX-260-036), was added to each EDTA blood tube (final concentration 40ul/ml plasma or 0.1mmol/ml). Addition of the DPP-4 inhibitor has been shown to prolong the $T_{1/2}$ to over 96 hours which will ensure preservation of the peptides for future analysis (Adaikalakoteswari et al. 2015b). Appendix 3.3 has more details on the preparation of Diprotin A, plasma tubes and blood collection protocol for this study.

The diagnosis of GDM was made according to the NICE 2015 criteria: fasting plasma glucose ($glucose_{0min}$) ≥ 5.6 mmol/l or 2-hour plasma glucose ($glucose_{120min}$) ≥ 7.8 mmol/l (National Institute for Health and Care Excellence 2015a). Women who tested positive were contacted and seen by the diabetes specialist midwife within 3 days of their GTT and were managed in a joint obstetric-diabetes antenatal clinic for the remainder of their pregnancy. Women whose GTT results were negative followed routine care.

### 8.2.4. Data collection and anthropometry measurement

Baseline demographic characteristics, anthropometric measurements and neonatal birth data were obtained from the parent PRiDE study database. A detailed medical history was recorded during recruitment into the study at 8 to 12 weeks gestation. Maternal weight was recorded using digital scales at baseline and during the GTT and gestational weight gain (GWG) between these 2 time-points calculated. Waist circumference was measured at baseline using a tape measure and triceps and subscapular skinfold thickness via calipers at both visits.

### 8.2.5. Laboratory analysis

Analysis of glucose was done by a hexokinase enzymatic method in the hospital laboratory. Plasma samples were stored at -80$^{o}$C until the end of the study when they were transferred to University of Copenhagen for analysis of GLP-1 on dry ice. A radioimmunoassay (RIA), which has been well-developed and previously described by Professor Jens Holst and colleagues, was used for the analysis of GLP-1 (Orskov et al. 1994; M.-B. Toft-Nielsen et al. 2001a). Briefly, the amidated forms of GLP-1, namely the bioactive GLP-1 (7-36) and its N-terminal truncated metabolite GLP-1 (7-37) were measured against standards of synthetic GLP-1 (7-36) using antibody (code number 89390) (M.-B. Toft-Nielsen et al. 2001a). Since these standards are specific for the amidated C-terminus of the hormone, they do not cross react with other GLP-1 containing peptides from the pancreas. It is essential to determine the concentrations of both intact GLP-1 (7-36) and the inactive (7-37) metabolite because of the rapid degradation of the former. The detection limit of the assay is 1pmol/l and intra-assay and inter-assay coefficient of variation less than 6% at 20 pmol/l and 10% respectively.

### 8.2.6. Statistical analysis

There have been 2 studies which compared GLP-1 AUC between GDM and NGT population (Table 7.1, discussed in more detail below) (Bonde et al. 2013; Lencioni et al. 2011). The first one found a non-significant 25% lower mean AUC in GDM compared to NGT women (Bonde et al. 2013). However, this was measured at a 4

hour liquid meal test which is not easily reproducible in a clinical setting. The other study found a non-significant 10.4% lower mean AUC in GDM than NGT women during a 100g 3-hour GTT(Lencioni et al. 2011).

To demonstrate a 25% lower mean AUC for GLP-1 in GDM compared to control with 80% power at 5% significance (2-tailed), the estimated sample size is 20 cases. In the PRiDE study, the detection rate of GDM was around 15% during GTT so I will planned to recruit 133 women into the study during their GTT.
Total area under the curve (AUC) for GLP-1 was calculated using the trapezoidal rule and incremental AUC calculated as the change from baseline GLP-1 levels.

Statistical analysis was performed using SPSS version 22.0 (IBM Corp Released 2013). BMI, and glucose values were log-transformed for statistical purposes as they were no normally distributed. For comparison of baseline characteristics of GDM and NGT women, the Student's t-test was used for continuous variables (e.g. BMI, age, gestational weight gain) and the chi-square test for categorical variables. Comparison of detailed anthropometry, glucose variables and GLP-1 parameters between cases and controls was done by analysis of variance (ANOVA) after adjusting for confounders. Stepwise multiple linear regression was performed with $glucose_{120min}$ as the dependent variables with the predictors entered or removed from the model according to the following criteria: probability of F Probability-of-F- to-enter $\leq 0.050$, Probability-of-F-to-remove $\geq 0.100$. The regression models included the following co-variates: age, BMI, ethnicity and smoking as standard. Where appropriate, other variables such as fasting glucose and gestation of bloods when evaluating associations of $glucose_{120min}$ or $GLP_{0min}$ when $GLP_{30min}$ was entered as a predictor of $glucose_{120min}$ were added to the models.

### 8.2.7. Ethics approval

Ethical approval from the National Research Ethics Committee (South Birmingham) (see Appendix 3.4) and permissions from the local Research and Development departments of the 2 NHS trusts where the study was conducted were obtained prior to commencement of the study.

## 8.3. Results

### 8.3.1. Selection of GDM cases and controls

A total of 145 women were recruited into the study but one woman did not complete the GTT due to vomiting. Therefore data from 144 women, from whom complete glucose values at 0 and 120 minutes were available, was used for analysis. Their mean (±SD) age was 29.5 ± 4.7 years and mean first trimester BMI 32.5 ± 8.13 kg/m$^2$.

The selection of cases and controls for this study is summarised in Figure 8.1. The pre-study intention was to analyse GLP-1 response in the lowest and highest quartile for glucose$_{120min}$ (n=35 and n=38 respectively). However, I excluded 15 outliers with unexpectedly low glucose$_{120min}$ results (defined as the lowest 10$^{th}$ centile for glucose$_{120min}$, range 2.7 to 3.9 mmol/l) from further analysis. This was on the basis that there could have been a laboratory error in measuring the glucose values (since these results were obtained from routine measurements in the NHS Trusts) or the possibility that some of these women may have exaggerated insulin responses which may be pathological.

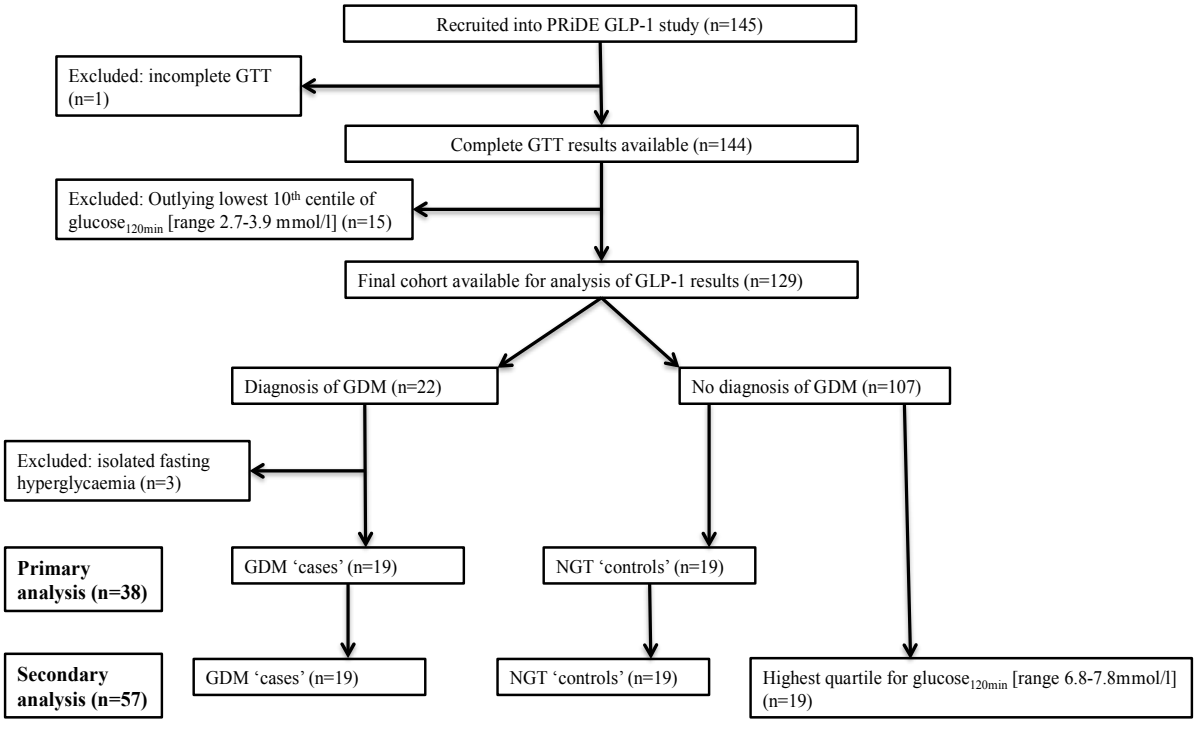


**Figure 8.1** Flow diagram illustrating the selection of gestational diabetes mellitus (GDM) and normal glucose tolerance (NGT) controls for the primary and secondary analyses of the study

Of the remaining 129 women, 22 women were diagnosed with GDM using the NICE 2015 criteria and their mean (±SD) fasting and 2-hour post-challenge glucose values were 5.15 ± 1.02 and 8.45 ± 2.00 mmol/l respectively. The corresponding GTT results from the remaining 122 women were 4.33 ± 0.33 and 5.40 ± 1.19 mmol/l respectively.

Out of the 22 cases of GDM diagnosed, 19 had $glucose_{120min}$ at or above the diagnostic threshold of 7.8 mmol/l and were labelled as 'GDM cases' for purposes of this study. The 0 and 120 minute glucose values of the other 3 women were 7.2 and 3.3 mmol/l, 5.9 and 4.9 mmol/l and 6 and 5.9 mmol/l respectively. These 3 women were not considered as having GDM for purposes of this study for the following 2 reasons: 1) isolated fasting hyperglycaemia in pregnancy is likely to have a different pathogenesis from the insulin resistance typically seen in GDM [(Chakera et al. 2015; S. J. Yang et al. 2013) and 2) the hypothesis of this study is that reduced GLP-1 response is responsible for impairment insulin secretion or action following a glucose load thereby leading to post-load hyperglycaemia.

The 19 cases of GDM had $glucose_{120min}$ values between 7.8 and 12.1 mmol/l. The controls were made up of 19 women in the lowest quartile with $glucose_{120min}$ values above 4.0 mmol/l (range of $glucose_{120min:}$ 4.0 to 4.5 mmol/l inclusive) and are referred to the normal glucose tolerance (NGT) group. The cut-off of 4.0 mmol/l was determined after excluding the outliers in the lowest $10^{th}$ centile as described above.

It has been shown in several studies that the morbidity associated with GDM is continuous with increasing glucose values with no clear threshold effect (Hapo Study Cooperative Research Group et al. 2008). This is partly the reason that there are several diagnostic criteria for GDM in use across the world with no definite consensus (International Association of et al. 2010; Meek et al. 2015). Thus, a secondary aim of this study was to measure how the GLP-1 response varied with $glucose_{120min}$ as a continuous variable. We measured the GLP-1 levels of the additional 19 women who were in the highest quartile of $glucose_{120min}$ values but did not meet the threshold for GDM diagnosis (range of $glucose_{120min}$: 6.8 to 7.7 mmol/l). Therefore, the total sample size used for the secondary GLP-1 analysis was 57.

### 8.3.2. Characteristics of GDM and NGT women at baseline and during GTT

The baseline characteristics of the 38 women (19 GDM, 19 NGT) included in the primary outcome analysis are presented in Table 8.1. This data was recorded in the late 1st trimester (mean gestation $12^{+1}$ weeks). The mean (±SD) age of the women was 29.0 ± 4.93 years, 13.9% were smokers and 81.6% of White Caucasian (or white European) origin, with no significant difference in any of these parameters between the 2 groups.

**Table 8.1** Baseline participant characteristics according to GDM status

| Variables | GDM | NGT | p-value |
|---|---|---|---|
| Number | 19 | 19 | |
| Age (yrs) | 29.5 ± 5.2 | 28.4 ± 4.7 | NS |
| Baseline weight | 100.1 ± 19.2 | 81.2 ± 16.6 | 0.003 |
| Body mass index (kg/m$^2$) | 37.6 ± 8.1 | 29.1 ± 5.3 | 0.001 |
| Triceps thickness (mm) | 30.0 ± 5.9 | 27.7 ± 8.1 | NS |
| Subscapular thickness (mm) | 32.7 ± 7.7 | 27.7 ± 8.2 | NS |
| Waist circumference (cm) | 115.6 ± 16.5 | 96.0 ± 12.9 | <0.001 |
| Current smokers (%) | 4 (21.1) | 1 (5.9) | NS |
| Ethnicity (%) | | | |
| European | 17 (89.5) | 14 (82.4) | NS |
| South Asian | 2 (10.5) | 2 (11.8) | |
| Afro-Caribbean | 0 | 1 (5.9) | |

Table showing the baseline characteristics and anthropometry of GDM and NGT women recorded in early pregnancy (mean ± SD gestation 85.2±14.5 days, $12^{+1}$ weeks). Continuous variables are mean ± SD, categorical variables are percentages. Comparisons done by Student's t-test (for continuous variables) or Pearson ci-square test (for categorical variables). GDM: gestational diabetes mellitus, NGT: normal glucose tolerance, NS: non-significant

**Table 8.2** Participant characteristics and glucose values according to GDM status during GTT

| Variables | GDM | NGT | p-value | Adj p-value[BMI, age, ethnicity, smoking] |
|---|---|---|---|---|
| Gestation of GTT (weeks) | $26^{\pm 6}$ ($15^{+6}$, $30^{+2}$) | $26^{\pm 6}$ ($15^{+6}$, $29^{+5}$) | NS | |
| Gestational weight gain (kg) | 3.44 ± 3.82 | 5.94 ± 3.31 | 0.05 | NS |
| Triceps thickness (mm) | 34.2 ± 4.5 | 27.4 ± 8.6 | 0.01 | NS [above + early pregnancy triceps thickness] |
| Subscapular thickness (mm) | 34.9 ± 4.5 | 26.1 ± 6.5 | <0.001 | NS [above + early pregnancy subscapular thickness] |
| $Glucose_{0min}$ (mmol/l) § | 4.95 ± 0.94 | 4.18 ± 0.27 | 0.002 | NS |
| $Glucose_{120min}$ (mmol/l) § | 9.04 ± 1.34 | 4.29 ± 0.15 | <0.001 | <0.001 [above +fasting glucose] |

Participant characteristics and anthropometry at recruitment into PRiDE GLP-1 study (during 75g 2-hour GTT). Data are mean (±SD) or median (range). Comparison of means by Student's t-test or ANOVA after adjustment for covariates (age, BMI, ethnicity, smoking and where appropriate triceps and subscapular skinfold thickness and $glucose_{0min}$). GTT: glucose tolerance test, GDM: gestational diabetes mellitus, NGT: normal glucose tolerance, NS: non-significant, BMI: body mass index

The women who went onto develop GDM had a higher baseline weight and BMI than the controls, which was not unexpected (100.1 ± 19.2 vs. 81.2 ± 16.6 kg, p=0.003 and 37.6 ± 8.1 vs. 81.2 ± 16.6 kg/$m^2$, p=0.001). The former group also had a higher waist circumference (115.6 ± 16.5 vs. 96.0 ± 12.9 cm, p<0.001) although other anthropometry (e.g. triceps and subscapular skinfold thickness) did not differ.

Both groups of women had their GTT (and GLP-1 blood sampling done) at a median of $26^{+6}$ weeks and the results are summarised in Table 8.2. Fasting glucose was marginally higher in the GDM group than NGT (4.95 ± 0.94 vs. 4.18 ± 0.27 mmol/l, p=0.002) but the difference lost significance after correcting for age, BMI, ethnicity and smoking. However the mean glucose level 2 hours after the GTT was significantly higher in the former group even after adjusting for co-variates, including fasting glucose (9.04 ± 1.34 vs. 4.29 ± 0.15 mmol/l, p<0.001). Other anthropometry (gestational weight gain, triceps and subscapular skinfold thickness) were all higher in the GDM group although any observed difference became insignificant after adjustment, likely due to the effect of BMI as a confounder.

### 8.3.3. GLP-1 profile of GDM and NGT women

The results of the GLP-1 measurements of GDM and NGT women at the time of GTT are presented in Table 8.3. The GLP-1 concentrations at 0, 30, 60, 90 and 120 minutes of the GTT are referred to as $GLP_{0min}$, $GLP_{30min}$, $GLP_{60min}$, $GLP_{90min}$ and $GLP_{120min}$ respectively.

**Table 8.3** Results of GLP-1 response during GTT according to GDM status

| Variables | GDM | NGT | p-value | Adjusted p-value |
|---|---|---|---|---|
| $GLP_{0min}$ (pmol/l) | 13.6 ± 4.46 | 15.7 ± 4.08 | NS | NS |
| $GLP_{30min}$ (pmol/l) | 15.9 ± 3.89 | 19.8 ± 4.52 | 0.01 | 0.041 |
| $GLP_{60min}$ (pmol/l) | 18.1 ± 4.96 | 20.6 ± 4.44 | NS | NS |
| $GLP_{90min}$ (pmol/l) | 18.3 ± 4.49 | 20.2 ± 4.84 | NS | NS |
| $GLP_{120min}$ (pmol/l) | 18.2 ± 4.61 | 18.8 ± 4.66 | NS | NS |
| Mean GLP (pmol/l) | 16.8 ± 3.33 | 18.8 ± 3.23 | NS | NS |
| Total AUC (pmol/l.120min) | 2024 ± 422 | 2321 ± 408 | 0.036 | 0.048 |
| Incremental AUC (pmol/l.120min) | 384 ± 464 | 427 ± 474 | NS | NS |

Participant characteristics and anthropometry at recruitment into PRiDE GLP-1 study (during 75g 2-hour GTT). Data are mean (±SD), comparison of means by Student's t-test or ANOVA after adjustment for covariates (age, BMI, ethnicity and smoking). GTT: glucose tolerance test, GDM: gestational diabetes mellitus, NGT: normal glucose tolerance, NS: non-significant, AUC: area under the curve

Mean $GLP_{0min}$ concentrations did not differ between the 2 groups. However GDM women had 19.7% lower mean $GLP_{30min}$ concentrations than NGT women, which remained significant even after adjusting for age, BMI, ethnicity and smoking (15.9 ± 3.89 vs. 19.8 ± 4.52 pmol/l, p=0.01 and adjusted p=0.04) (Table 8.3, Figure 8.2). The former group had a tendency to lower GLP-1 levels at 60, 90 and 120 minutes as well although none of these results reached statistical significance.

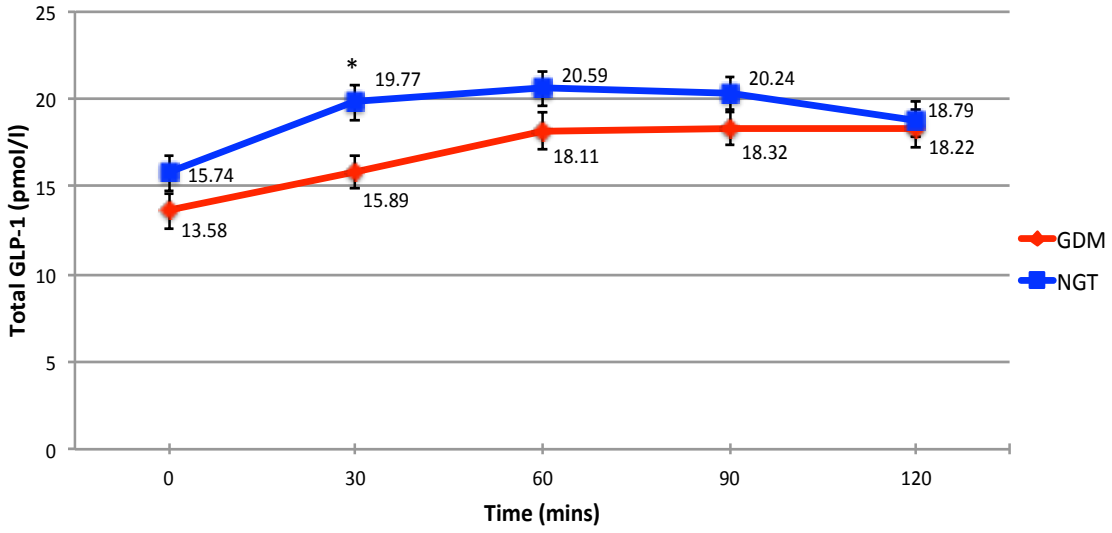


**Figure 8.2** Line chart of the plasma GLP-1 concentrations during a glucose tolerance test (GTT) in women diagnosed with gestational diabetes mellitus (GDM, red diamonds) and normal glucose tolerance (NGT, blue squares). The points represent the mean (±SEM) plasma GLP-1 concentration measured at 30 minute intervals during a 2-hour 75g GTT. *: $p<0.05$

Other markers of the GLP-1 response which were calculated from the individual levels at the 5 time points were the mean overall GLP-1 concentration, total AUC for GLP-1 response and incremental AUC from basal. These results are presented in Table 8.3 and Figure 8.3 (a) – (c). The overall GLP-1 response to a glucose challenge as measured by total AUC was 12.8% lower in GDM women than controls, which weakened but still remained significant after adjusting for confounders (2024 ± 422 vs. 2321 ± 408 pmol/l.120min, p=0.036, adjusted p=0.048). The other 2 parameters measured were non-significantly lower in GDM than NGT.

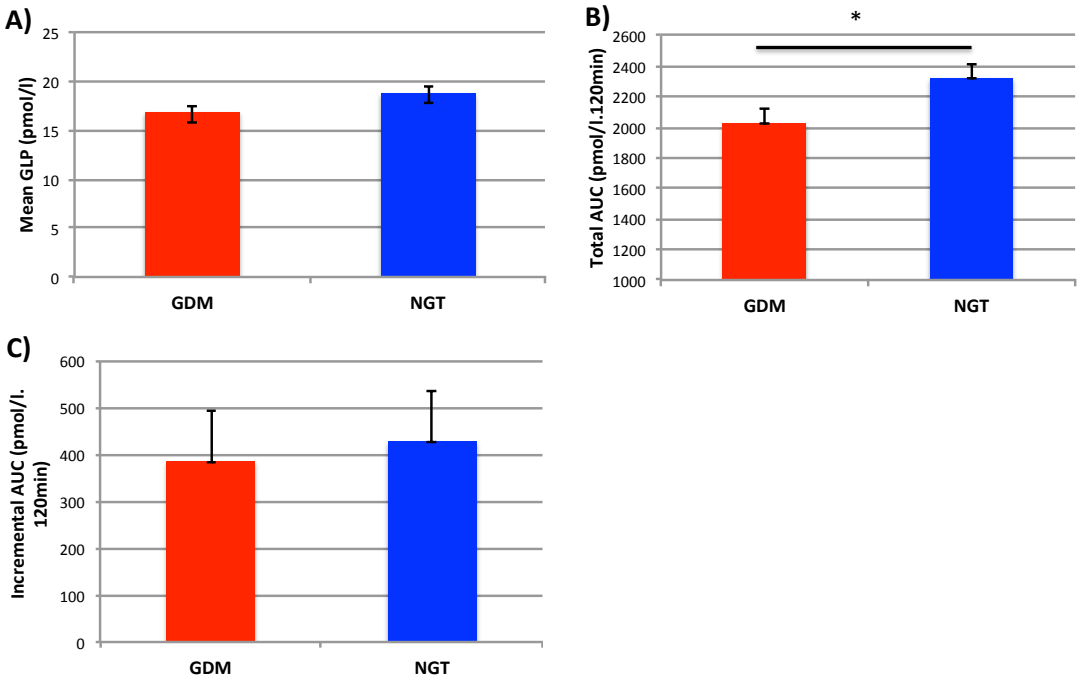


**Figure 8.3** Bar charts of 3 markers of GLP-1 response during a glucose tolerance test (GTT) in pregnant women diagnosed with gestational diabetes mellitus (GDM) and those with normal glucose tolerance (NGT). A) Mean of 5 GLP-1 levels measured at 30 minute intervals during a 2-hour GTT; B) total area under the curve (AUC) of GLP-1 response calculated using trapezoidal rule; C) incremental AUC calculated as AUC above basal. Data are mean + SEM. *: $p<0.05$

### 8.3.4. GLP-1 as a predictor of glucose$_{120min}$

To determine if the GLP-1 response reduces linearly with increasing glucose levels, we considered a larger cohort of 57 women who had all the relevant parameters analysed. On bivariate correlation, glucose$_{120min}$ was negatively associated with GLP$_{30min}$ and total AUC (Pearson's r -0.29, p=0.04 and -0.34, p=0.01 respectively) (data not shown). However there was no association with GLP$_{0min}$, GLP$_{60min}$, GLP$_{90min}$ and GLP$_{120min}$ or mean GLP-1 levels. There was also no correlation with any of the above GLP-1 parameters and glucose$_{0min}$ confirming that GLP-1 only influences glycaemic control in response to an oral glucose load.

The next step was to examine if the negative associations between glucose$_{120min}$ and the GLP-1 variables remained after correcting for confounders. The results of the multiple linear regression analysis with glucose$_{120min}$ as the dependent variable are presented in Tables 8.4 (a) to (c). In a model with total AUC, only that and glucose$_{0min}$ predicted glucose$_{120min}$ after adjusting for age, BMI, ethnicity, smoking and gestation of GTT (Table 8.4 (a)). However, when gestational weight gain was

added to the model, the former lost significance. A similar observation was found for $GLP_{0min}$ with the exception that the association strengthened after adjusting for gestational weight gain (β-coefficient -0.25, p=0.04 and p=0.02 in the 2 models respectively) (Table 8.4 (b)). In the regression analysis with $GLP_{30min,}$ $GLP_{0min}$ was also added as a co-variate because I was interested in looking at $GLP_{30min}$ as an independent variable. The results confirmed the independent negative association with $glucose_{120min,}$ with a stronger effect size than the abovementioned 2 GLP-1 parameters (β-coefficient -0.31, p=0.02 before and after adding gestational weight gain into the model). GLP-1 levels at 60, 90 or 120 minutes were not associated with $glucose_{120min}$ in any of the models and neither was mean GLP-1 levels (data not shown).

**Table 8.4 (a) - (c)** Multiple regression analysis of predictors of $Glucose_{120min}$ using various GLP-1 parameters as co-variates[1]

**a) Total AUC**

| **Predictors of $Glucose_{120min}$ §** | **B-coefficient[2]** | **Standardised β[2]** | **p-value[3]** | **p-value[4]** |
|---|---|---|---|---|
| $Glucose_{0min}$ § | 1.18 | 0.49 | < 0.001 | < 0.001 |
| Total AUC | $-8.40^{-5}$ | -0.25 | 0.047 | 0.08 |
| Age | - | - | NS | NS |
| BMI § | - | - | NS | NS |
| Ethnicity | - | - | NS | NS |
| Smoking | - | - | NS | NS |
| Gestation of GTT | - | - | NS | NS |

**b) $GLP_{0min}$**

| **Predictors of $Glucose_{120min}$ §** | **B-coefficient** | **Standardised β** | **p-value[3]** | **p-value[4]** |
|---|---|---|---|---|
| $Glucose_{0min}$ § | 1.25 | 0.52 | <0.001 | <0.001 |
| $GLP_{0min}$ | -0.01 | -0.25 | 0.039 | 0.021 |
| Age | - | - | NS | NS |
| BMI § | - | - | NS | NS |
| Ethnicity | - | - | NS | NS |
| Smoking | - | - | NS | NS |
| Gestation of GTT | - | - | NS | NS |

**c) $GLP_{30min}$**

| **Predictors of $Glucose_{120min}$ §** | **B-coefficient** | **Standardised β** | **p-value[3]** | **p-value[4]** |
|---|---|---|---|---|
| $Glucose_{0min}$ § | 0.96 | 0.40 | 0.003 | NS |
| $GLP_{30min}$ | -0.01 | -0.31 | 0.017 | 0.016 |
| BMI § | 0.53[5] | 0.43[5] | NS | 0.002 |
| Age | - | - | NS | NS |
| Ethnicity | - | - | NS | NS |

| | | | | |
|---|---|---|---|---|
| Smoking | - | - | NS | NS |
| Gestation of GTT | - | - | NS | NS |
| $GLP_{0min}$ | - | - | NS | NS |

Tables showing multiple linear regression results of the predictors of Glucose120min using 3 parameters of GLP-1 measurements as co-variates: a) Total AUC, b) $GLP_{0min}$ and c) $GLP_{30min}$

1: sample size is n=57 from the total cohort who had complete GTT and GLP-1 results measured

2: unstandardised B and standardised β-coefficients are with reference to model 3 unless stated otherwise

3: Model includes $glucose_{0min}$, BMI, age, ethnicity, smoking, gestation as additional co-variates (plus $GLP_{0min}$ for the $GLP_{30min}$ analysis)

4: As for Model 3 + gestational weight gain

5: Unstandardised B and standardised β-coefficients are from model 4

§: log-transformed for statistical comparison, -: tested but not significant in the model, NS: non-significant, BMI: body mass index, GTT: glucose tolerance test

### 8.3.5. Variables associated with thresholds of GLP-1 parameters

Since there is no data on what constitutes an adequate GLP-1 response or optimal levels, I was interested to find out if there were any factors associated with different thresholds of GLP-1. I selected $GLP_{30min}$ and total AUC to examine this since they were the 2 which had the strongest link to glycaemia in the previous analyses. These women were divided into 2 groups based on the median of these 2 parameters and the variables compared between the women with $GLP_{30min}$ and total AUC below and above the median are shown in Tables 8.5 (a) and (b). $Glucose_{120min}$, was significantly higher in women total AUC and $GLP_{30min}$ in the lower half of the distribution confirming the inverse relationship between them. Lower total AUC levels seemed to have a stronger relationship with impaired post-prandial glycaemia on the basis that the difference remained significant after adjusting for confounders. Other maternal factors, including fasting glucose, BMI, waist circumference, skinfold thickness and gestational weight gain did not show any associations.

**Table 8.5 (a) - (b)** Maternal anthropometry and glucose results according to thresholds of GLP-1 parameters[1]

**a) Total AUC split by median**

| | Total AUC below median | Total AUC above median | p-value | Adj p-value[2] | Adj p-value[3] |
|---|---|---|---|---|---|
| $Glucose_{0min}$ § | 4.68 ± 0.611 | 4.51 ± 0.78 | NS | NS | NS |
| $Glucose_{120min}$ § | 7.43 ± 1.98 | 6.12 ± 2.17 | 0.015 | 0.037 | NS |
| BMI ($kg/m^2$) § | 32.9 ± 7.33 | 33.5 ± 8.14 | NS | NS | NS |
| Waist circumference (cm) | 106.2 ± 16.23 | 105.9 ± 16.86 | NS | NS | NS |
| Triceps skinfold thickness (mm) | 26.9 ± 7.25 | 29.6 ± 6.97 | NS | NS | NS |
| Subscapular skinfold thickness (mm) | 29.8 ± 9.84 | 29.7 ± 8.66 | NS | NS | NS |
| Gestational weight gain (kg) | 4.7 ± 4.27 | 5.1 ± 3.22 | NS | NS | NS |

**b) $GLP_{30min}$ split by median**

| | $GLP_{30min}$ below median | $GLP_{30min}$ above median | p-value | Adj p-value[2] | Adj p-value[3] |
|---|---|---|---|---|---|
| $Glucose_{0min}$ § | 4.65 ± 0.51 | 4.50 ± 0.79 | NS | NS | NS |
| $Glucose_{120min}$ § | 7.25 ± 1.55 | 6.32 ± 2.34 | 0.041 | NS | NS |
| BMI ($kg/m^2$) § | 34.5 ± 8.38 | 32.7 ± 8.39 | NS | NS | NS |
| Waist circumference (cm) | 109.1 ± 16.31 | 104.5 ± 18.35 | NS | NS | NS |
| Triceps skinfold thickness (mm) | 27.3 ± 7.63 | 29.7 ± 6.69 | NS | NS | NS |
| Subscapular skinfold thickness (mm) | 30.2 ± 9.51 | 29.1 ± 8.70 | NS | NS | NS |
| Gestational weight gain (kg) | 4.1 ± 4.29 | 5.8 ± 3.40 | NS | NS | NS |

Table of maternal anthropometry (recorded in early pregnancy) and glucose results (from GTT) in participants according to thresholds of (a) total AUC of GLP-1 and (b) $GLP_{30min.}$ Data are mean (±SD), comparison of means by Student's t-test or ANOVA after adjustment for covariates.

1: sample size is n=57 from the total cohort who had complete GTT and GLP-1 results measured

2: Model adjusted for BMI, age, ethnicity and smoking

3: As for model 2 plus gestational weight gain

§: log-transformed for statistical comparison, AUC: area under the curve, NS: non-significant, BMI: body mass index, GTT: glucose tolerance test

## 8.4. Discussion

*Main findings*

The analysis of GLP-1 profile in GDM and hyperglycaemia of pregnancy reveal 3 key findings:- 1) Overall GLP-1 response as measured by total AUC reduced by 13% in GDM compared to NGT pregnancies; 2) There seems to be an early impairment or 'delay' in GLP-1 secretion during the GTT in GDM (as shown by 20% lower $GLP_{30min}$ levels) and 3) Lower GLP-1 concentrations at baseline or 30 minutes after a consuming glucose load independently predict higher glucose levels at 2 hours.

*Comparison with other studies in GDM*

3 of the 6 studies which investigated GLP-1 response or the incretin effect during GDM pregnancy reported mean and/or total AUC for GLP-1 (see summary table in Chapter 7, Table 7.1) (Bonde et al. 2013; Cypryk et al. 2007; Lencioni et al. 2011). Of these, *Bonde et al* found a non-significant decrease of 25% in the 3$^{rd}$ trimester of pregnancy compared to NGT. This result is in keeping with that found by our study but the magnitude of difference we found was lower at 13%. Possible reasons for this variance are that 1) a smaller sample size (the study was powered to detect a difference in GLP-1 response in GDM women between pregnancy and post-partum), 2) different glucose load used and 3) longer duration of sampling (4 hours vs. 2 hours in our study). It has been shown previously that mixed meal load is a stronger stimulus for incretin release (but not GLP-1) than an isolated glucose load possibly because of its higher fat and protein content (Vollmer et al. 2008).

The studies by *Lencioni et al* and *Cypryk et al* found no difference in the total response of GLP-1 of GDM women during a 3-hour 100g and 75g GTT respectively (Cypryk et al. 2007; Lencioni et al. 2011). In the *Lencioni et al* study, mean GLP-1 values were lower only at 180 minutes in GDM compared to NGT (37.1 vs. 27.6 pmol/l, $p<0.05$). However it should be noted that the authors only sampled GLP-1 at 0, 60, 120 and 180 minutes of the GTT thereby any potential lowering of GLP-1 at 30 minutes, which was observed in our cohort, would have been missed. This study reported lower first phase insulin secretion and the related insulin sensitivity-secretion index in GDM women (discussed in detail in the following section) but this should be interpreted with caution because the authors of the original formula have stated that the optimal sampling time to estimate first phase insulin secretion should include

insulin data at 30 minutes (Stumvoll et al. 2001). Therefore it may not be possible to draw any conclusions from this study about the relationship between the early GLP-1 impairment, which was observed in our study and impaired insulin secretion.

Lencioni and colleagues also did not find any correlation between GLP-1 AUC and maternal factors such as age or BMI, which was in keeping with our results. Previous studies which have found a positive relationship between GLP-1 concentrations and BMI in non-pregnant adults have measured GLP-1 levels across a range of BMIs (E. Muscelli et al. 2008a; M.-B. Toft-Nielsen et al. 2001a). Therefore the result cannot necessarily be extrapolated to our cohort of pregnant women since the majority of them were obese due to the selective screening criteria used for GTT although the GDM women were heavier (mean BMI 37.6 vs. 29.1 $kg/m^2$ in GDM and NGT women respectively). In our study, there was no correlation between BMI or other surrogate markers of obesity (e.g. waist circumference, skinfold thickness) and any of the GLP-1 indices.

Fasting GLP-1 levels in GDM pregnancies continues to be controversial. While *Bonde et al* found this to be nearly 30% lower in glucose intolerant women, our study found a non-significant 13% lowering. However, Cypryk and colleagues and Reyes-Lopez and colleagues both noted significantly higher fasting GLP-1 in GDM women (Cypryk et al. 2007; Reyes-Lopez et al. 2014). Type 2 diabetic patients have lower fasting GLP-1 levels, which was in turn associated with higher baseline insulin concentrations and HbA1c, regardless of the duration of diabetes (Legakis et al. 2003). Interestingly, higher fasting GLP-1 levels may be metabolically protective in glucose tolerant adults where it is positively associated with resting energy expenditure and substrate oxidation (Pannacciulli et al. 2006). Taken together, these findings suggest that fasting GLP-1 concentrations are expected to be decreased in GDM pregnancies and the finding by *Cypryk et al* and *Reyes-Lopez et al* may be outliers.

*Kosinski et al* did not measure GLP-1 levels in their study of 10 GDM women and 8 NGT controls but did detailed studies (4-hour GTT and isoglycaemic intravenous glucose infusion) to measure the incretin effect and gastrointestinal-induced glucose disposal (GIGD) (Kosinski et al. 2013). They showed that the incretin effect as

measured by AUC for insulin and C-peptide were not reduced in GDM compared to NGT mothers. However GIGD, which is a measure of the amount of glucose preferentially cleared by the oral route (largely by activating incretin hormones) (J. J. Holst et al. 2016) were reduced to levels similar to diabetic patients in GDM (37±3 vs. 48±3%, p=0.04).

The 5th study by *Gonzalez et al* only measured a random GLP-1 concentration during delivery in 15 GDM women (Gonzalez et al. 2011) so their findings cannot be directly compared with those of my study and others in pregnancy which looked at the response of GLP-1 to an oral glucose or mixed meal load. Additionally, this was the only study to measure GLP-1 by an electrochemiluminescent assay (ELISA), which detects only the active GLP-1 forms (namely 7-36 and 7-37). Therefore, timely addition of DPP-4 inhibitors is crucial to prevent degradation of active GLP-1. A comparative study has shown relatively weak correlation between the ELISA technique and the RIA method used in our study ($r=0.39$, $p<0.05$) (Heijboer et al. 2011).

*Consequences of impaired early phase insulin secretion in non-pregnant adults*

A novel finding of my study is a particular time point of lower GLP-1 response (i.e. $GLP_{30min}$) seems to be linked to adverse outcomes, namely decreased levels in GDM vs. NGT after adjustment for confounders and additionally as an negative predictor of post-challenge glucose as a continuous variable. The latter observation was found in a range of $glucose_{120min}$ values below the threshold of GDM diagnosis. I will now discuss the implications of impaired early phase GLP-1 secretion and its potential relevance to impaired insulin secretion in impaired glucose tolerance (IGT) and GDM.

First-phase insulin secretion is typically defined as the acute insulin response after an intravenous glucose load but because that can be cumbersome to perform, various calculations such as the Stumvoll formulae have been developed and validated to estimate it after an oral glucose load. The Stumvoll calculation takes into consideration fasting glucose and insulin at 0 and 30 minutes to estimate the early insulin response (Stumvoll et al. 2000). Impairment in the first phase of insulin secretion has been shown to be one of the earliest detectable abnormalities in adults

who are predisposed to develop T2DM (Gerich 2002; Weyer et al. 1999) and independently predicts conversion from NGT to T2DM (Y. Sun et al. 2016). The decrease in first-phase insulin secretion may be due to a reduction in beta-cell mass due to ageing or genetic factors (Szoke et al. 2008; Vaag et al. 1995). However, in adults with IGT, it has been shown to be compromised to a greater degree than the second phase insulin secretion during a hyperglycaemic clamp experiment (Szoke et al. 2008), suggesting that it be involved in the primary pathogenesis of insulin resistance

The insulinogenic index, defined as the incremental change in insulin concentrations at 30 minutes of a GTT divided by the change in glucose at 30 minutes in the same time period is an surrogate of the early insulin response and beta-cell function (Wareham et al. 1995). In a large study of over 5000 Japanese adults, the insulinogenic index of people with isolated IGT was significantly lower than those with isolated impaired fasting glucose (IFG) and it was the strongest independent predictor of $glucose_{120min}$ in patients with IGT (**β**-coefficient -0.29, $p<0.0001$) while $glucose_{0min}$ was predicted by the insulin sensitivity index (Aoyama-Sasabe et al. 2016). In a separate study, plasma insulin levels at 30 minutes following an oral glucose load was negatively correlated with glucose concentrations at 2-hours supporting the relationship with IGT ($r=-0.75$, $p<0.001$) (Mitrakou et al. 1992).

Animal studies have shown that the first phase insulin secretion has a greater effect on reducing gluconeogenesis from the liver than peripheral disposition in skeletal muscles, providing further insight into how its impairment may contribute to IGT or T2D (Del Prato and Tiengo 2001; Steiner et al. 1986).

It has been proposed that diabetes or pre-diabetes caused by predominant IGT is a different pathophysiological entity from predominant IFG. The former has been shown in epidemiological studies to have greater association with future cardiovascular-related deaths (Decode Study Group 2001; Tominaga et al. 1999). Tissue level studies confirm these findings since acute hyperglycaemia can lead to development of diabetic complications mediated by a hypercoagulable state, impairment of endothelial function and oxidative stress (Del Prato and Tiengo 2001).

*Insulin indices in GDM*

Whilst there have been several studies investigating the precise insulin secretion and resistance abnormalities in T2D or IGT (as discussed above), the evidence in GDM is more sparse. There is conflicting data on whether GDM is predominantly a problem of insulin resistance or impaired pancreatic beta-cell function whereby insulin secretion cannot be increased in response to rise in glucose. Studies that have supported the former abnormality have found higher fasting insulin levels, HOMA-IR and weaker correlation between fasting glucose and C-peptide in GDM (Ismail et al. 2013; Weijers et al. 2002). In fact, *Ismail at al* showed that higher HOMA-IR scores were even predictive of neonatal hypoglycaemia and Caesarean section rates in GDM, raising the possibility that it could be the main pathophysiological factor in mediating complications. However other studies have shown that fasting insulin levels are lower in GDM compared to NGT women (suggesting preserved insulin sensitivity) and concluded that the predominant problem is defective **β** –cell function (R. L. Mamabolo et al. 2007).

Specifically the insulinogenic index, which is a surrogate marker of early phase insulin secretion (as discussed above) was reduced by 35-60% in GDM women in mid- or late-pregnancy compared to NGT (R. L. Mamabolo et al. 2007; Y. H. Wang et al. 2013). However in the study by *Mamabolo et al*, when the insulinogenic index was adjusted for changes in insulin sensitivity, the result became non-significant suggesting that the 2 abnormalities are not mutually exclusive entities. In a different study, impairment in the early phase insulin secretion correlated negatively with higher amplitude of glucose excursion and higher overall insulin secretion, so it may also be a marker of the development of insulin resistance in pregnancy (Su et al. 2013). Impairment of insulin secretion found in women diagnosed with GDM or milder glucose intolerance was associated with a decrease in post-partum **β**-cell function (as measured by insulin secretion-sensitivity index) at 3-12 months after pregnancy but increased in previous NGT women, suggesting long-term metabolic effects (Retnakaran et al. 2010b).

The reality is that GDM is most likely a combination of both impairment in insulin secretion and resistance with increase in HOMA-IR and reduction in insulin sensitivity index, first and second phase insulin secretion and HOMA**β**- cell function

(S. J. Yang et al. 2013). Interestingly, even in a subgroup of women with milder gestational IGT (i.e. below the threshold for diagnosis of GDM) all the above indices, except for HOMA-IR but including the insulin sensitivity index and insulin-secretion sensitivity index, were significantly different compared to NGT lending support for the findings of my study that the metabolic insults to these women occur across a continuum of glucose values. It has also been shown that a decrease in insulin sensitivity occurs physiologically in all pregnant women but when insulin secretion does not increase sufficiently to compensate for this, hyperglycaemia or GDM occurs (Damm et al. 1996).

Although ours was a case-control study where the controls were selected from the lowest quartile for $glucose_{120min}$ levels, fasting glucose levels were also higher in GDM compared to NGT women [4.95 vs. 4.18 mmol/l, $p<0.01$], supporting earlier findings that insulin resistance plays a role in the pathogenesis of the condition. We have not analysed the insulin concentrations (collected at the same 5 time-points as GLP-1) in our study yet, which will add value to the findings as it will allow calculation of surrogates of insulin resistance as well (e.g. HOMA-IR, insulin sensitivity index, etc.).

*Potential role of GLP-1 in mediating defective early phase insulin secretion*

Given the evidence supporting the link between early impairment in insulin response and overall **β** –cell function and tendency to glucose intolerance, it is plausible that factors which affect early insulin secretion, such as reduced GLP-1 response in the initial period of the GTT, will increase tendency to IGT too.

There have been no previous studies which have shown an impairment of GLP-1 response at a specific time-point of a GTT and development of GDM. However, in a series of women with previous GDM and NGT controls who underwent a 75g GTT at 4-5 years after the index pregnancy, the former group had lower initial insulin and GLP-1 responses (measured as 0 – 30 minute AUC for both) despite normalisation of glucose values, which can be postulated to be involved in their higher risk of progression to T2D (Forbes et al. 2005). This finding was not replicated in another cohort of women studied following GDM pregnancy (Meier et al. 2005).

The inverse relationship between $GLP_{30min}$ concentrations (or early GLP-1 response between 0 and 30 minutes) and insulin secretion abnormalities or impaired glucose tolerance in non-pregnant adults has been better characterised. Early impairment in insulin secretion during GTT has been shown in adults with IGT, which were associated with or may be caused by decreased GLP-1 levels at 30 minutes (Rask et al. 2004). More recently, the large ADDITION-PRO study found equivocal results in that T2D women had a 1.2-fold decrease in early GLP-1 response compared to isolated IFG but no difference was found in IGT women or all male participants (Faerch et al. 2015). This study also found that a doubling of the relative GLP-1 response between 0 and 30 minutes was associated with a 23% rise in the insulinogenic index but only 4% rise in the insulin sensitivity index suggesting that it influences **β**-cell function more than amelioration of insulin resistance. However, this observation is not universal because a multi-national study found overall decreased GLP-1 levels during intravenous GTT in all patients with abnormal glucose tolerance, with no relation to differential insulin secretion (Laakso et al. 2008).

The effect of GLP-1 in augmentation of first phase insulin secretion has been confirmed by hyperglycaemic clamp studies whereby infusion of duodenal nutrition augmented first phase insulin secretion more than second, which was then attenuated by infusion of a GLP-1 receptor antagonist, exendine-(9-39) (Woerle et al. 2012).

Significant insight into the mechanism by which GLP-1 influences insulin secretion is additionally provided by studying diabetic patients who have undergone bariatric surgery. One series of 30 patients found that diabetes remission after roux-en-y bypass was related to enhanced insulin secretion and exaggerated post-prandial GLP-1 response in the early phase, thereby suggesting an association between the two (Martinussen et al. 2015).

Therefore, although the relationship between lower GLP-1 levels at 30 minutes GTT and impaired early phase insulin secretion after a GTT has not yet been shown in my study (due to unavailability of insulin levels), it may be present, based on the findings from IGT or T2D adults, thereby providing a novel mechanism to explain the pathogenesis of GDM.

*Strengths and weaknesses*

This is the largest study, to our knowledge, investigating GLP-1 levels in GDM pregnancy using a glucose challenge that is routinely available clinically (i.e. the 75g oral GTT). The study has significant advantages in that at the time of GLP-1 sampling, neither the pregnant women now the research team knew her GDM status, thereby minimising selection bias. Additionally, GLP-1 levels were sampled every 30 minutes over 2 hours which enabled us to obtain a detailed profile of GLP-1 changes during the GTT and observe finer differences at the various time points.

However, there are some important limitations which cannot be ignored. Firstly, the matching of cases and controls was done according to the highest and lowest quartiles for $glucose_{120min}$ values, thereby potentially exaggerating differences in GLP-1 levels or response. However, since the thresholds for diagnosing GDM are variable across different countries and one of our aims was to detect differences in GLP-1 response across a continuum of glucose values, this was thought to be the best method. Additionally, we adjusted for potential confounders of post-prandial hyperglycaemia in our analysis, such as BMI, age, ethnicity, and even fasting glucose and gestational weight gain where appropriate, which would remove some of the inherent differences between women with highest and lowest $glucose_{120min}$ values. Secondly, although the required sample size was 20 women with GDM based on the power calculation for total GLP-1 response, we only considered 19 for this analysis and excluded 3 who had isolated fasting glycaemia. This may partly account for the reason why the magnitude of difference seen in total GLP-1 response was weaker than expected. Thirdly, to prove or disprove the hypothesis that a lower early GLP-1 response may account for early impairment in insulin secretion, insulin levels for the duration of GTT and glucose levels at 30, 60 and 90 minutes are required (which have been collected but not analysed yet). Finally, although we accounted for several confounders that can influence risk of GDM or GLP-1 levels, there are other variables (eg. parity, socioeconomic status) which were not adjusted for in the regression analysis. This information has been collected as a part of the PRiDE study and will be analysed and included in any further data generated from this pilot study.

### 8.5. Conclusion

In summary, we have shown that total GLP-1 response is reduced by 13% in GDM women compared to NGT following a GTT and, for the first time in a pregnancy cohort, that GLP-1 levels at 30 minutes are reduced by 20% in GDM and that they independently predict higher glucose values at 120 minutes. If the association with lower early phase insulin secretion is proven with further analysis, our study will provide novel evidence that impairment of GLP-1 secretion may be responsible for the decrease in early phase insulin response found in glucose intolerance of pregnancy.

# Chapter 9

# Mechanism of action of liraglutide in the alleviation of hyperglycaemia-induced oxidative stress in endothelial cells: methodology of a basic science project

## 9.1. Introduction

Longitudinal studies have shown that women with a history of gestational diabetes mellitus (GDM) are 7.4 times higher risk of developing type 2 diabetes (T2D) (Bellamy et al. 2009). Although the risk increases with longer follow-up times, the pooled risk ratio was still 4.7 at less than 5 years from the index pregnancy. Additionally, these women are at are also at increased risk of metabolic syndrome and cardiovascular complications in the future, which accounts for most of the excess morbidity and mortality in diabetes (Retnakaran et al. 2010a; Schalkwijk and Stehouwer 2005). A recent 10-year follow up recent study in women with a history of GDM found increased hazard ratios of macro vascular complications (e.g. coronary artery disease) which were mediated by development of T2D but microvascular complications (e.g. photocoagulation for retinopathy, renal dialysis and foot infections) were independent of T2D status (Retnakaran and Shah 2017). It is well known that the abovementioned complications are mediated by endothelial dysfunction in the vasculature in response to hyperglycaemia. Indeed, endothelial dysfunction, as measured by flow mediated dilatation (FMD) of a peripheral artery, has been shown to be decreased in women with previous hyperglycaemia of pregnancy despite return to normoglycaemia (Anastasiou et al. 1998; Davenport et al. 2012).

Therefore, pregnancy provides a crucial opportunity to identify these high risk women and offer safe and effective therapeutic interventions to reduce future morbidity. In the previous chapter, I have shown that reduced glucagon like peptide-1 (GLP-1) response mediates post-glucose challenge hyperglycaemia in GDM pregnancies so it is plausible that GLP-1 analogues may have a protective role to play in reducing hyperglycaemia and associated complications. It is also possible that a sub-group of women who are identified to have impaired GLP-1 response to be given GLP-1 analogue post pregnancy to reduce their risk of developing recurrent GDM or subsequent future risk of T2D.

Most of the studies investigating the action of GLP-1 receptor agonists (GLP-1 RAs) on endothelial cells have attributed its benefits to enhanced endothelial nitric oxide

synthase (eNOS) production and consequent nitric oxide (NO)-mediated vasodilatation and anti-inflammatory effects (Ban et al. 2008; Dong et al. 2013). However, other mechanisms such as inhibition of endothelin-1 (a potent vasoconstrictor) (Dai et al. 2013) and advance glycation-end product (AGE) formation (Ishibashi et al. 2010) may also be present. It is not known which exact downstream pathways this class of drugs modulates in the context of hyperglycaemia-induced oxidative stress, but may involve the protein kinase B second messenger (also known as Akt), which is a potent activator of eNOS in the endothelial cell (Uruno et al. 2005).

The hypothesis of this set of experiments is that liraglutide will alleviate hyperglycaemia induced oxidative stress in endothelial cells via the Akt/eNOS and other related signaling pathways.

The primary aim of my pilot study was to describe a protocol for the cell culture of endothelial cells under high glucose (HG) conditions and determine optimal time point, concentration and duration of treatment with liraglutide to alleviate markers of oxidative stress. The overall objective of related experiments would be to determine if the drug protects against hyperglycaemia-induced endothelial cell damage by mediating by the Akt/eNOS signaling pathways or if other pathways (e.g. (AMPK) were predominantly involved. Prior to investigating this, I wanted to determine the time point of maximal Akt suppression after exposure to high glucose (HG) conditions and what the optimal concentration and duration of liraglutide treatment was as this has not yet been established in other studies.

## 9.2. Materials and Methods

### 9.2.1. Justification of study design

I used a cell culture model of human umbilical vein endothelial cells (HUVECs) which were exposed to HG (25mM) for variable lengths of time followed by

treatment with liraglutide. The proteins of interest were extracted at the end of cell culture experiments and their expression analysed by Western blotting. This was thought to be the best method to address my study questions since I was primarily interested in looking at the effect of oxidative stress generated in endothelial cells due to HG conditions, which will mimic the pathophysiology of cardiovascular complications in diabetic patients. Additionally I was interested in studying the mechanisms by which liraglutide has protective effect in these patients so a basic science model to examine its influence on the proteins of interest was felt to be the most appropriate.

### 9.2.2. Cell culture

#### 9.2.2.1. HUVEC cell culture: General principles

HUVECs were isolated from umbilical cords of patients from University Hospital of Coventry and Warwickshire after written informed consent. For all my experiments, I used previously extracted HUVECs that were cryopreserved in liquid nitrogen at passage 4-6. The cells were maintained in freezing media containing 10% dimethylsulfoxide (DMSO) (Fisher-Scientific), supplemented with 40% fetal bovine serum (FBS) (Biosera, Ringmer, UK) and 50% growth media.

Normal growth media for the cells contained 500ml MCDB 131 cell culture media (Gibco Life Technologies, UK) supplemented with 5ml glutamine, 5ml penicillin/streptomycin (Invitrogen, Paisley, UK), 20% or 10% (50mls) fetal bovine serum, 10mg (1ml) endothelial cell growth supplement from bovine neural tissue (Sigma-Aldrich, UK) and 8000 units (0.9ml) heparin (Sigma-Aldrich, UK). This was in keeping with other studies, which used similar protocols for the culture of HUVECs (Bruno Schisano et al. 2012).

All the cells were initially incubated in a 75ml flask containing 15ml growth media at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$ and 95% air. They were grown to 80-90% confluence before being subcultured into plates for further experiments. This was done by first washing with 15ml phosphate-buffered saline at room temperature

and 2.5mls of trypsin was added to ensure detachment. The cells were then centrifuged at 2000rpm for 15 minutes and the supernatant removed before the cells were resuspended in growth media.

A haemocytometer was used to count the cells and an average cell count of 1.2 x $10^6$ cells/ml media was obtained across all the experiments. From this, the cells were seeded at a density of 50,000 cells/well in gelatine-coated 6-well plates to achieve an initial confluence of 20-30%. At this seeding density, the cells usually reached confluence within 5 to 7 days when the experimental conditions were started.

Growth media was changed every 48 hours until the day before any treatments were due to be done, in which case the cells were exposed to serum starved media (normal growth media containing 3% FBS) overnight prior to changing to a normal glucose or high glucose media (see below).

When the cells were ready to be harvested at the various time-points or at end of the experiment, triplicates of protein and RNA were obtained from each plate. The cells were first washed 3 times with phosphate buffered saline (PBS). 1x radio-immunoprecipitation assay (RIPA, Sigma-Aldrich, UK) solution was made up by diluting 10x RIPA with distilled water. A cocktail of protease inhibitors was prepared, which contained 2 tablets of Roche Complete Mini, sodium fluoride 8mg and sodium orthovanadate 20mg added to 2ml of 1x RIPA. 100µl of the cocktail was mixed with 5ml of 1x RIPA and 250µl of this solution was added to each well at 4$^o$C prior to scraping each well for 30 seconds. For RNA extraction, a Bioline kit (#BIO-65026) was used and 350µl of this was added to each well with the rest of the procedure the same as that described for protein extraction. The cell lysates were then frozen at -20$^o$C until the time of future experiments.

#### 9.2.2.2. Gelatin-coating of flask

In order to optimize adherence of the cells to the flask, I first did an experiment of coating the flasks with gelatin. 10mls 0.2% gelatin was added to a 75cm$^2$ flask,

removed after 10 minutes and the flask allowed to dry for at least 30 minutes in an incubator. One cryovial of HUVECs was then added to the gelatin-coated flask containing 15mls growth media with a control set of cells in a flask without gelatin-coating. The cells in both sets were 20-30% confluent at the beginning. After 4 days of incubation, the cells in the gelatin coated flask were 50% confluent while those in the control flask had attached poorly. Therefore it was decided to continue all future experiments with gelatin coating of flasks and 6-well plates. The procedure for gelatin-coating the latter was similar to that described above, with the exception that 2ml gelatin was added to each well of the plate.

#### 9.2.2.3. Initial HUVEC culture (no treatment)

The aim of my first set of experiments was to optimize the cell culture conditions and practice the extraction of protein and RNA for future experiments. Two cryovials of HUVECs from liquid nitrogen were cultured in 2 75cm$^2$ flasks and after the 2$^{nd}$ passage, split into 4 x 6-well plates. Once the cells reached around 90% confluence, they were harvested and protein and RNA extracted according to the protocol described in Section 9.2.2.1.

#### 9.2.2.4. Chronic (10 day) high glucose treatment (with insulin stimulation)

One of the key research questions I had was whether exposure of endothelial cells to chronic HG would lead to inhibition of the phospho-Akt pathway due to the development of insulin resistance and if this inhibition could be alleviated by liraglutide. Therefore, this experiment was done to determine the time-point in which maximal phospho-Akt suppression or other markers of endothelial dysfunction occurs and to confirm if any inhibition was due to the development of insulin resistance.

To prepare the HG media, 20mM of 45% glucose (Sigma-Aldrich, UK) was added to 500ml of the earlier prepared normal glucose (NG) growth media (since MCDB 131 media already contains 5.5mM glucose). Therefore the final concentration of HG

media was 25mM glucose.

In keeping with other studies done on HUVECs, which used L-glucose for osmotic balance in the NG conditions (Busik et al. 2008), I cultured 2 additional plates with this condition at Day 2 of the experiment (termed normal glucose/L-glucose, NG/LG). Therefore there were a total of 5 time-points (between Days 2 and 10 of treatment) and for each time-point of the experiment, an additional plate exposed to each experimental condition had 100nmol/L of insulin added for 10 minutes prior to harvesting (termed NG+I, HG+I and NG/LG+I respectively).

To ensure optimal uptake of insulin by the cells, it was necessary to starve them of serum prior to insulin treatment. This was achieved by reducing the serum concentration of media by from 20% to 10% overnight on the day before the insulin treatment. On the day of the insulin treatment, the media was changed to NG, HG or NG/LG plus 1% bovine serum albumin for 3 hours before the insulin treatment was given and cells harvested.

### 9.2.3. Liraglutide treatment experiments

#### 9.2.3.1. Chronic (6 day) HG treatment

The aim of this experiment was to determine if liraglutide would alleviate hyperglycaemia-induced oxidative stress in HUVECs and the following cell culture conditions were used to determine the optimal time-point, concentration and duration of treatment of Liraglutide.

HUVECs were cultures as described in Section 9.2.2.1 and transferred to 6-well plates at a seeding density of 50, 000 cells per well when over 80% confluence was reached. On the day prior to insulin or liraglutide treatment, the cells were kept in a serum starved HG medium (containing 3% FBS).

Liraglutide was obtained as from Novo Nordisk as a complement. A stock of 6mg/ml in a 3ml vial was received on dry ice and from this 3 concentrations (1nM, 10nM and 100nM) were prepared and frozen until further use. On the day of the experiment, liraglutide was added to serum-starved media and the cells in the HG media treated with each of these concentrations of liraglutide for 3 or 6 hours prior to harvesting on Days 2, 4 and 6. The control groups consisted of NG, NG+LG and HG plates (without liraglutide treatment).

#### 9.2.3.2. Acute (12, 24 and 48 hours) HG treatment

Since previous studies done on HUVECs treated with liraglutide or other GLP-1 analogues did so after a shorter duration of HG exposure due to accelerated cell apoptosis (Batchuluun et al. 2014), I hypothesised that the maximal benefit of Liraglutide may be seen after acute exposure to hyperglycaemia.

The aim of this experiment was to confirm if maximal downregulation of phospho-Akt and phospho-AMPK expression in endothelial cells occurred at less than 48 hours of exposure to hyperglycaemia and whether treatment with liraglutide alleviated this. Cells were exposed to HG conditions for 12, 24 and 48 hours. Similar to the earlier experiment, they were treated with Liraglutide at concentrations of 1, 10 or 100nM for 3 or 6 hours. However, to avoid any bias from unequal duration of exposure to HG (due to the shorter duration of treatment overall), additional control plates of NG and HG were harvested at 3 and 6 hours in line with the duration of liraglutide treatment. The cell culture and harvesting protocol was identical to previous experiments.

### 9.2.4. Protein quantification

In order to quantify the RIPA extracted proteins for further experiments, 1µg/µL Bovine Serum Albumin (BSA, Sigma, UK) was added to 1ml Bradford Protein Assay

kit (Biorad) in serial dilutions from 0 to 7µL. This was used to determine the standard curve of absorbance against protein concentration. A volume of 5µL of the protein samples was added to the Bradford reagent and both the standards and samples were measured at a wavelength of 595nm using a spectrophotometer (Tecan, UK).

### 9.2.5. Western blot

The composition of the materials used for the Western blot experiments (e.g. running and transfer buffers, Tris-buffered saline, I-block and sodium dodecyl sulphate (SDS) gels are provided in Appendix 4.1).

Protein lysates were prepared by adding the 15ug of protein to 10ul of loading dye and distilled water to make the total volume up to 40 or 50ul. They were heated at 95$^{o}$C for 10 minutes and then cooled on ice before being centrifuged at 10000 rpm for 15 seconds to ensure even suspension.

The samples were loaded onto 10% polyacrylamide gels (Geneflow Ltd, Fradley, UK) and separated by SDS-PAGE at 100V for 2 hours. They were then transferred to SDS-PAGE membranes, blocked with I-Block for 1 hour and incubated with primary antibodies (+0.2% I-block) overnight. The next day, the membranes were washed 6 times for 5 minutes each with Tris-buffered saline under gentle agitation. Following this anti-rabbit secondary antibody was added at a concentration of 1:50 000 (+0.2% I-Block), followed by 6 washes with Tris-buffered saline. The ECL/ECL+ chemiluminescent detection system (GE Healthcare, Amersham Biosciences, UK) was added before the membranes were subject to X-ray imaging for visualisation. Densitometry was done to quantify the intensity of the bands (GeneTool Software, Syngene, UK).

The primary antibodies and their concentrations are as follows: phospho-Akt (Ser473)

(1:500, Cell Signaling), phospho-AMPK (1:500, Cell Signaling), phospho-eNOS (Ser1177) (1:250, Cell Signaling), Bcl-2 associated X protein (BAX) (1:1000, Cell Signaling), phospho-forkhead box class O 1a (phospho-FOXO 1a) (1:500, Cell Signaling) and β-Actin (1:1000, Cell Signaling).

In all the Western blot experiments, equal protein loading was confirmed by normalisation was with β–Actin (1:1000, Cell Signaling). Stripping buffer was prepared dissolving 0.76g Tris base, 2g SDS and 700ul in 100ml β-mercaptoethanol. The pH was adjusted to 6.8 and the solution warmed to 50$^{o}$C in an incubator before the rehydrated membranes were added to it. The membranes were placed in the stripping buffer for 5 to 10 minutes under gentle agitation then washed 8 times before being blocked with 0.2% I-block. The remainder of the steps were identical to that of the in Western blot (Section 9.2.5).

### 9.2.6. Statistical analysis

All statistical analysis was performed using SPSS version 22 (IBM Corp Released 2013). The results are expressed as mean ± SD of 3 experiments, unless otherwise stated. Paired student's t-test was used to compare means of control and treated samples. The significance value was set as $P < 0.05$ for all experiments.

### 9.2.7. Ethics approval

The HUVECs used for the experiments were isolated from umbilical cords provided by pregnant women at University Hospitals Coventry and Warwickshire NHS Trust at the time of delivery. Written informed consent was obtained from all women and after local ethics committee approval.

## 9.3. Results

### 9.3.1. Cell culture and Western blot technique optimisation experiments

For the initial experiments, I cultured the HUVECs in NG conditions without any treatment. Figure 9.1 shows the cells at Day 2 after reviving (around 20% confluence). The cells grew in a monolayer with a typical cobblestone appearance and the individual cells were spindle-shaped.

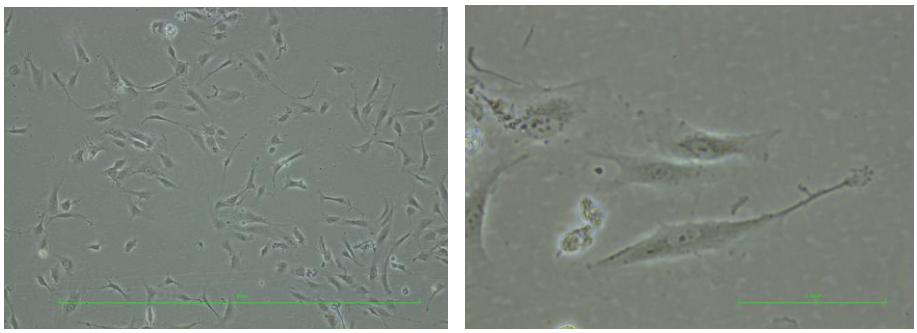

**Figure 9.1 Microscope image HUVEC cells at A) 10x (left hand image) and B) 40x magnification (right hand image)**. In this experiment, the cells were cultured under normoglycaemic (5.5mM) conditions with MCDB-131 growth medium.

The first few western blots which I did were on the untreated samples from the above experiment to optimise the protocol and antibody concentrations. Before incubating with the primary antibody, each membrane was typically cut at the 95kDa mark so that so that at least 2 proteins of interest could be studied, phospho-eNOS (molecular weight, MW 140 kDa) and phospho-Akt or phospho-AMPK (MW 60 and 64 kDa respectively). Figure 9.2 is a photograph of 2 membranes immersed in Ponceau solution, to visualize the proteins and aid accurate cutting the membranes.

**Figure 9.2 Photograph of 2 SDS-PAGE membranes from a western blot immersed in Ponceau stai**n. The proteins were separated according to their molecular weight and visualised against the markers in lanes 1 and 10. They were then cut at predetermined molecular weights and added to the appropriate primary antibody.

Despite using 2 different stripping buffers and reprobing protocols on several Western blot experiments, I was unable to obtain convincing results for the total isoforms of the various phosho-proteins studied. The bands, which were of the same molecular weight as the phospho-proteins, were not visualised after reprobing. Therefore, equal protein loading was studied by reprobing for beta-actin (molecular weight 42 kDa) and the results for protein expression in the following sections are presented after normalisation with actin.

### 9.3.2. 10 day HG experiment with insulin

*Phospho-Akt (Ser473)*

There was a trend of decrease in Akt (Ser473) phosphorylation with HG conditions compared to NG at Days 2 and 4 but it did not reach statistical significance (Figure 9.3). At Day 6, phospho-Akt expression decreased by 15% with HG compared to NG control at the same time point ($p<0.05$). However, the same trend was not observed at Days 8 and 10. On visualisation of the cells under microscopy at the time of cell culture, increased cell death noted at these time-points as well.

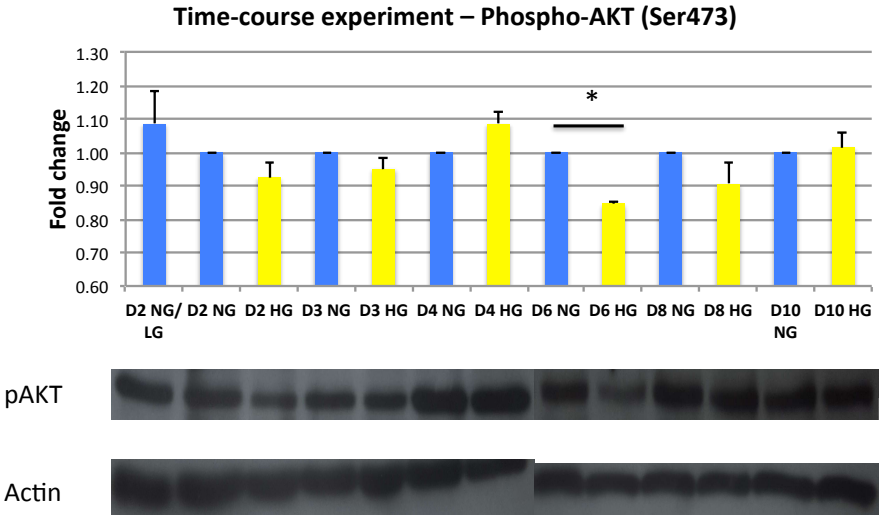


**Figure 9.3 Time-course of phospho-AKT (Ser473) expression after exposure to high glucose (HG).** Human umbilical endothelial cells were cultured in 6-well plates. Upon confluence, they were exposed to HG conditions (25 mM) for 2, 3, 4, 6, 8 and 10 days (abbreviated as D2/3/4/6/8/10 respectively). Control plates were in normal glucose (NG) or NG + L-glucose (NG/LG, 20mM) for osmotic balance. Phospho-AKT (Ser473) expression was determined by Western blot. The graph is that of fold-change of the protein, against NG for that respective day, after normalising with β-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figures. *: $p<0.05$

*Phospho-eNOS (Ser1177)*

There was slight decrease in phospho-eNOS (Ser1177) expression found at Days 2 and 6 with exposure to HG conditions (Figure 9.4). However none of the results were significant enough to draw any meaningful conclusions.

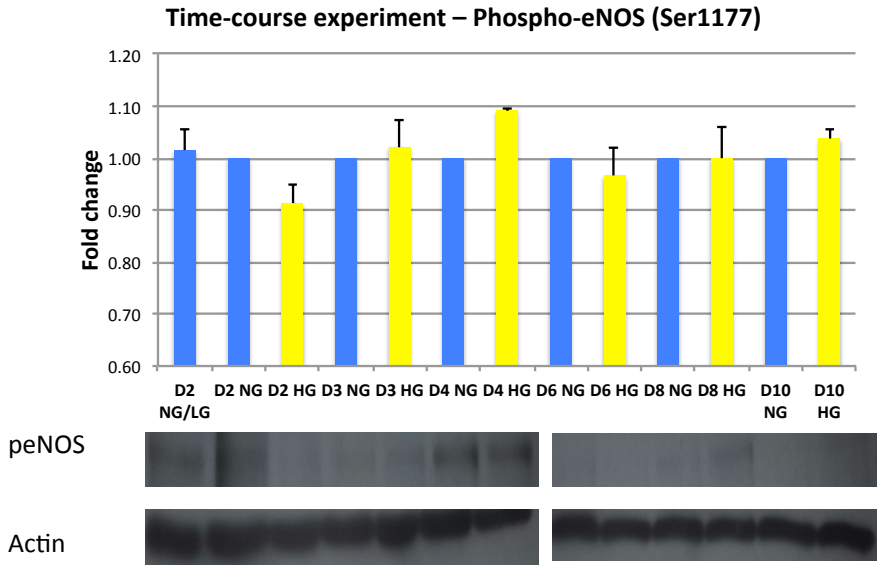


**Figure 9.4 Time-course of phospho-eNOS (Ser1177) expression after exposure to high glucose (HG).** Experimental details and abbreviations are as provided in Figure legend 9.3. The graph is that of fold-change of the protein, against NG for that respective day, after normalising with B-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figures.

*Insulin stimulated experiment*

Two independent experiments done on 6 separate days of treatment (Days 3, 4, 6, 8 and 10) did not show any increase in phospho-Akt expression following insulin treatment compared to the respective HG conditions without insulin (data not shown). Therefore it was concluded that phospho-Akt activation in response insulin acting on insulin-receptor substrate (IRS) does not play a major role in endothelial cells (compared to other tissues such as skeletal muscle and adipose tissue and no further experiments with insulin stimulation were done (Artwohl et al. 2007)).

### 9.3.3. Liraglutide treatment after 48 hours exposure to HG

From the first set of liraglutide experiments, I decided to test the samples treated at 48 hours (Day 2) because there is evidence that endothelial cells undergo apoptosis at exposure to hyperglycaemia for more than 48 hours (Hou et al. 2015). Therefore it will not be clear if any observed elevation in markers of cell damage or oxidative stress are due to hyperglycaemia or apoptosis.

*Phospho-Akt (Ser473)*

Western blots from 3 independent experiments showed no significant change in phospho-Akt (Ser473) expression after 48 hours of treatment with 25mM glucose before or after adding liraglutide 1, 10 or 100nM (Figure 9.5).

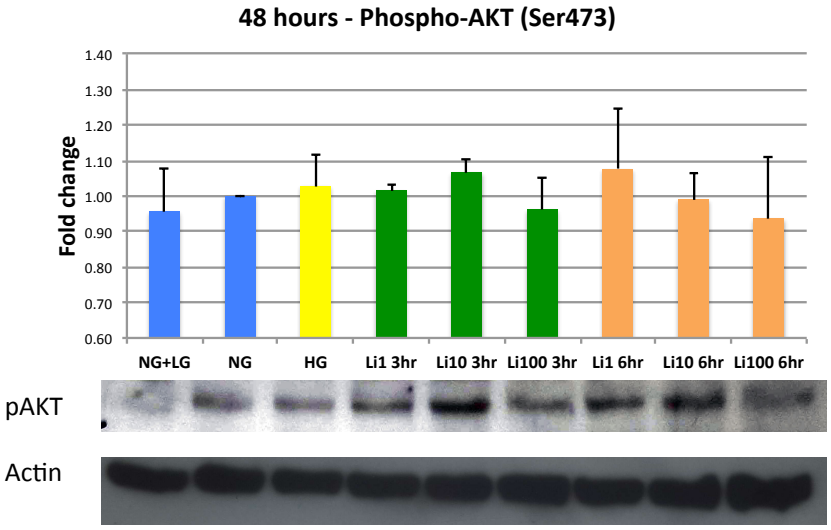


**Figure 9.5 Expression of phospho-AKT (Ser473) in endothelial cells exposed to high glucose (HG) for 48 hours followed by Liraglutide treatment.** Human umbilical endothelial cells were cultured in 6-well plates. Upon confluence, they were exposed to HG conditions (25 mM) for 48 hours and treated with Liraglutide 1nM, 10nM or 100nM for 3 hours (abbreviated as Li1 3hr/Li10 3hr/Li1003hr) or 6 hours (Li 1 6hr/Li10 6hr/Li100 6hr). Control plates were in normal glucose (NG) or NG + L-glucose (NG/LG, 20mM) for osmotic balance. Phospho-AKT expression was determined by Western blot. The graph is that of fold-change of the protein, against NG, after normalising with β-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure.

*Phospho-AMPK*

Three independent experiments confirmed that phospho-AMPK was downregulated after exposure to 48 hours of hyperglycaemia (Figure 9.6). Although there was not much change in suppression of phospho-AMPK after 3 hours of treatment of liraglutide, it became upregulated at 6 hours which was significant with 1nM liraglutide (fold-change compared to HG 1.68, $p<0.05$). There was also alleviation of phospho-AMPK with 10nM liraglutide at 6 hours but this did not reach statistical significance.

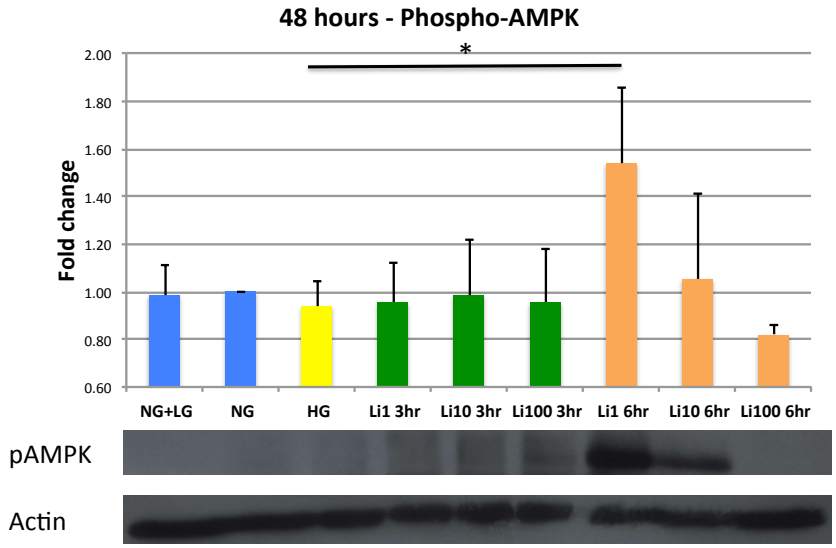


**Figure 9.6 Expression of phospho-AMPK in endothelial cells exposed to high glucose (HG) for 48 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure. *: $p<0.05$

*Phospho-eNOS (Ser1177)*

As expected, phospho-eNOS (Ser1177) expression was downregulated after exposure to HG. The alleviation of eNOS (Ser1177) suppression by liraglutide mirrored that of phospho-AMPK, with maximal upregulation with 1nM of drug at 6 hours although the difference did not reach statistical significance (Figure 9.7).

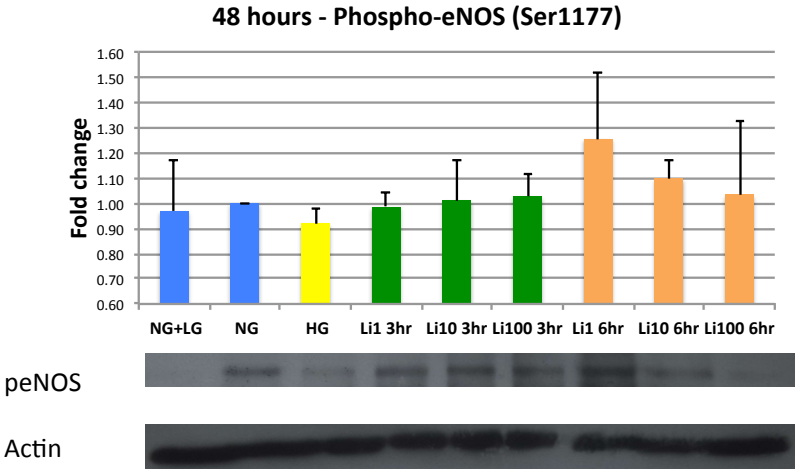


**Figure 9.7 Expression of phospho-AMPK in endothelial cells exposed to high glucose (HG) for 48 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin Two independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure

*BAX*

BAX is a pro-apoptotic protein which has been shown to induce caspase-3, a key enzyme involved in cellular apoptosis (Finucane et al. 1999). Bcl-2 and BAX form a heterodimer complex and decreased Bcl-2/BAX ratio, which may be caused by low Akt expression, leads to apoptosis (Hou et al. 2015; Oltvai et al. 1993).

Therefore, I examined BAX expression under the experimental conditions in my study. BAX expression was upregulated with 25mM glucose exposure but treatment with 100mM of liraglutide for 6 hours reduced this marginally by 1.06-fold ($p<0.05$) (Figure 9.8).

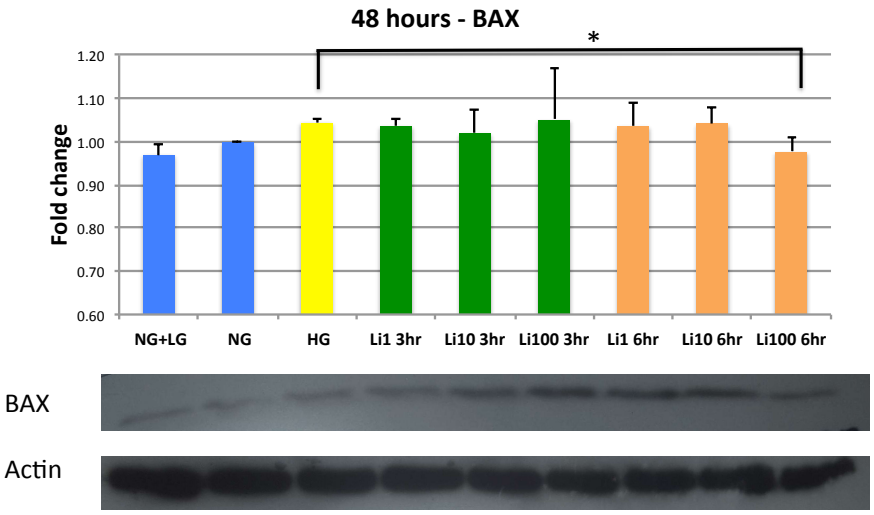


**Figure 9.8 Expression of BAX in endothelial cells exposed to high glucose (HG) for 48 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure. *: $p<0.05$

*Phospho-FOXO1a*

To confirm that suppression of Akt phosphorylation was not the major signaling mechanism causing cell damage after 48 hours of hyperglycaemia exposure or that Liraglutide did not exert any beneficial effect through this, I repeated the experiment with phospho-FOXO1, a direct substrate of phospho-Akt. Similar to the pattern observed with phospho-Akt, there was no change observed in phospho-FOXO1 expression with HG or subsequent liraglutide treatment (Figure 9.9)

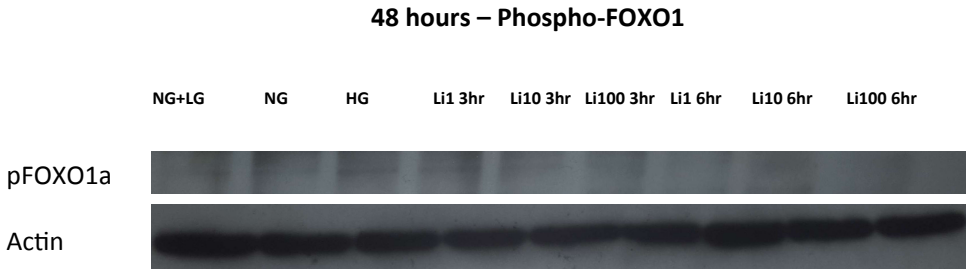


**Figure 9.9 Western blot of expression of phospho-FOXO1 after exposure to high glucose (HG) and subsequent Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. Results were only obtained from a single experiment due to technical problems with the antibody and detection of the protein.

### 9.3.4. Liraglutide treatment after 12 hours exposure to HG

Since other studies have shown cell damage with HG treatment for 12 hours and the inhibition of oxidative stress by liraglutide may be seen as early as 1 hour after treatment (Batchuluun et al. 2014), it was decided to repeat the cell culture experiments with 12, 24 and 48 hours of exposure to hyperglycaemia. I analysed the results after 12 hours of exposure to HG.

*Phospho-Akt(Ser473)*

Three independent experiments showed a marginal but significant alleviation of phospho-Akt suppression with 10nM liraglutide treatment for 3 hours (fold-change 1.06, $p<0.05$) (Figure 9.10).

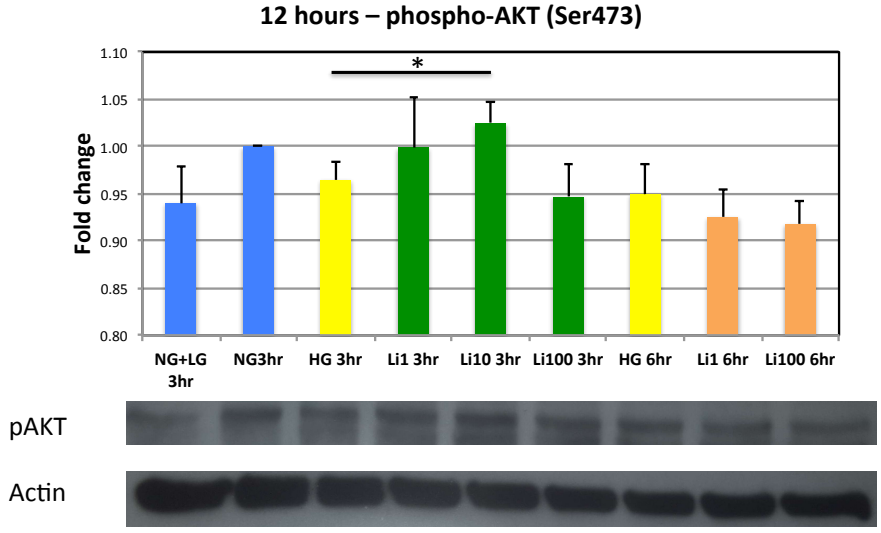


**Figure 9.10 Expression of phospho-AKT (Ser473) in endothelial cells exposed to high glucose (HG) for 12 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure. *: $p<0.05$

*Phospho-AMPK*

The effect of Liraglutide on phospho-AMPK was similar to that seen with phospho-Akt with the exception that a significant difference was observed with 1nM Liraglutide treatment for 3 hours instead of 10nM (fold-change 1.07, $p<0.05$) (Figure 9.11). There was no benefit noted with 6 hours of liraglutide treatment when the duration of hyperglycaemia exposure was only 12 hours.

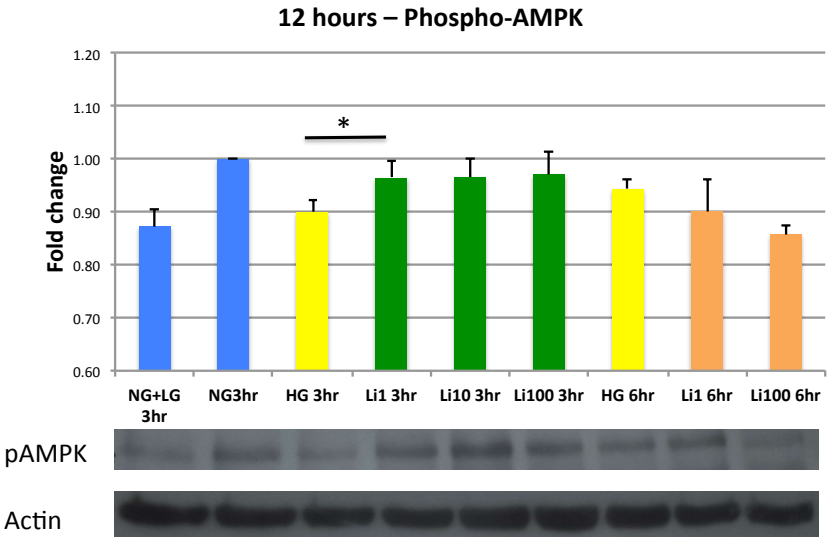


**Figure 9.11 Expression of phospho-AMPK in endothelial cells exposed to high glucose (HG) for 12 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure.. *: $p<0.05$

*Phospho-eNOS*

No difference in eNOS (Ser1177) phosphorylation was observed with liraglutide treatment at any dose or duration of treatment in these experiments (Figure 9.12).

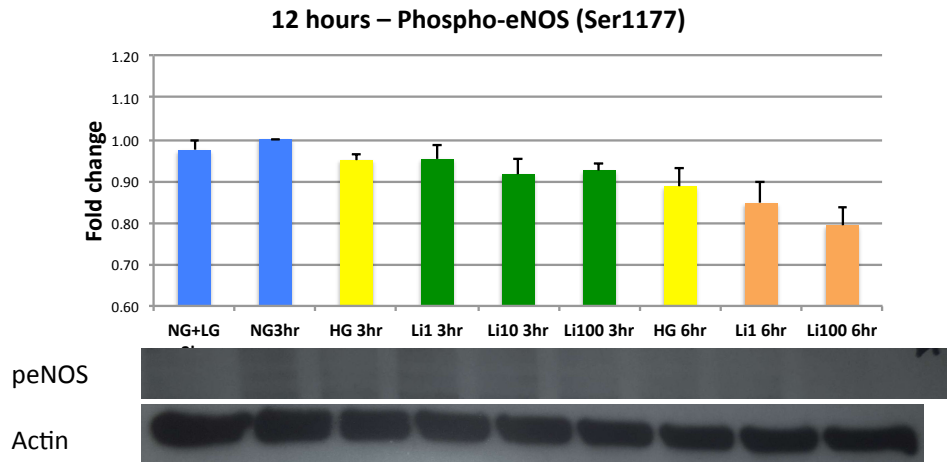


**Figure 9.12 Expression of phospho-eNOS (Ser1177) in endothelial cells exposed to high glucose (HG) for 12 hours followed by Liraglutide treatment.** Experimental details and abbreviations are as provided in Figure legend 9.5. The graph is that of fold-change of the protein, against NG, after normalising with β-actin. Three independent experiments were performed and data are mean ± SEM of these. Representative blot provided in the figure.

## 9.4. Discussion

The primary objective of this study was to develop a protocol for liraglutide treatment of endothelial cells exposed to HG conditions with the overall aim of studying the protective pathways it influences in the alleviation of oxidative stress. This was demonstrated by cell culture experiments using different time points of HG treatment, concentrations and durations of treatment of the drug followed by Western blots to look at the expression of proteins of interest. Based on preliminary data prior to normalising the expression of phospho-proteins with the total isoforms, 3 findings from my results are: (1) oxidative stress mediated cellular apoptosis in endothelial cells exposed to *48 hours of HG* is likely mediated by the phospho-AMPK pathway and phospho-Akt signaling does not appear to play a major role; (2) treatment with liraglutide for 6 hours can alleviate some of this damage by upregulating phospho-AMPK; (3) with more *acute (12 hours)* hyperglycaemia, inhibition of both phospho-Akt and phospho-AMPK pathways may be present and a shorter duration of liraglutide treatment (for 3 hours) upregulates both these proteins. There is no clear dose-dependent relationship for the effect of liraglutide, instead the duration of treatment seems to be proportional to the duration of exposure to hyperglycaemia.

*Signaling pathways activated by oxidative stress in hyperglycaemic conditions*

When I begun these experiments, my hypothesis was that liraglutide exerted its benefit on endothelial damage by reducing oxidative stress and the primary aim was to elucidate the mechanisms behind this. However, the first set of experiments were not conclusive of a definite suppression of Akt phosphorylation in HG conditions, with the exception of the Day 6 time-point (Figure 9.3). Possible reasons for this lack of effect are that the duration of HG exposure was too long causing senescence and apoptosis (a previous experiments has shown reduced cell viability with even 15mM of glucose after 24 or 48 hours (Hou et al. 2015)). An alternate explanation is that there is no significant effect on the phosphoinositol-3 kinase (PI3K)/Akt pathway by hyperglycaemia in endothelial cells. Glucose uptake and metabolism in response to insulin, which are important effects of Akt activation in muscle cells and adipocytes, are not shown to be activated by high glucose and insulin in micro- and macro-vascular endothelial cells (Artwohl et al. 2007) so it is possible that Akt activation by insulin receptor substrate is not a major player in this cell type.

Hyperglycaemia can activate oxidative stress and endothelial dysfunction by several pathways (reviewed in Kolluru and colleagues, Figure 9.13)(Kolluru et al. 2012). Briefly, these include uncoupling of eNOS and defective Akt/eNOS phosphorylation, which may be as a consequence of insulin resistance (McVeigh et al. 1992; Pannirselvam et al. 2002), activation of the diacylglyeryl (DAG)/protein kinase C (PKC)/NADH oxidase and nuclear factor-kappa B (NF-κB) pathways and formation of AGE. Therefore I sought to determine which of these pathways were alleviated by liraglutide treatment in my experiments.

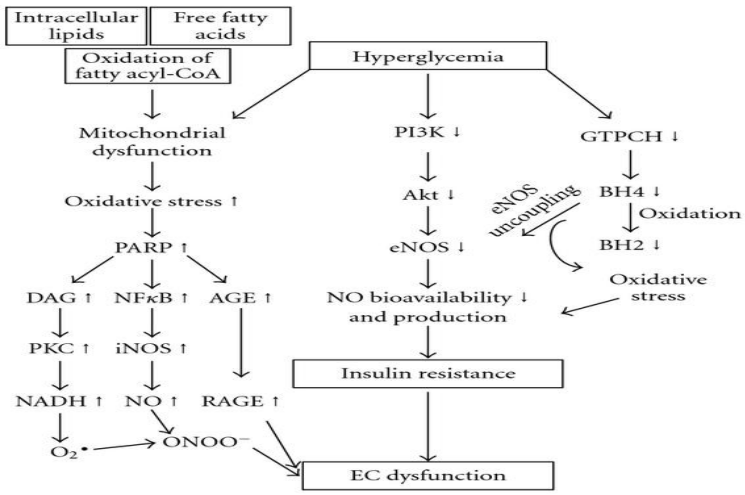


**Figure 9.13 Signaling mechanisms leading to endothelial dysfunction in diabetes mellitus.** pARP: poly (ADP-ribose) polymerase; AGE: advance glycation end products; DAG: diacylglycerol; NF-κB: nuclear factor kappa- B; PKC: protein kinase C; iNOS: inducible nitric oxide synthase; NADH: nicotinamide adenine dinucleotide; NO: nitric oxide; RAGE: receptor for advanced glycation endproducts; O2 • : super- oxide anion; ONOO− : peroxynitrite; PI3K: phosphatidylinositol 3-kinases; AKT: protein kinase B; eNOS: endothelial nitric oxide synthase; GTPCH: GTP cyclohydrolase; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin. (From Kolluru et al. 2012)

*Anti-oxidant effects of Liraglutide*

Although it is well recognized that liraglutide functions as an antioxidant, there are various theories on how this occurs. They include promotion of vasodilatation due to upregulation of eNOS levels, reduction in endothelin-1 or preventing cellular damage by other means (Dai et al. 2013; Ding and Zhang 2012). Indeed, the protective effect of GLP-1 on endothelial cells have been shown to occur by NO-dependent and independent means (Ban et al. 2008; Nystrom et al. 2004). GLP-1 also alleviates oxidative damage by suppressing AGE receptor expression and consequent reduction in AGE-mediated vascular-cell adhesion molecule-1 (VCAM-1) (Ishibashi et al. 2010).

It is important to note that although all these processes of endothelial damage can occur with hyperglycaemia, the abovementioned experiments by other groups were conducted under normoglycaemic conditions. Therefore it is possible that exposure to HG and consequent cell damage may activate additional pathways, which liraglutide has an effect on.

*Effect of Liraglutide on the AMPK pathway*

My study showed that with moderate (i.e. 48-hour) hyperglycaemia exposure, liraglutide exerts its benefit predominantly on the AMPK pathway, which leads to inhibition of cellular apoptosis (reflected by downregulation of BAX expression) (Figures 9.6 and 9.8). The effect on AMPK phosphorylation occurred maximally after 6 hours of treatment with 1nM liraglutide. Downregulation of BAX, albeit with a much lower magnitude of effect, also occurred with liraglutide treatment for 6 hours suggesting that the anti-apoptotic benefits may be due to AMPK signaling. Liraglutide had no effect on phospho-Akt or phospho-eNOS expression at the 48-hour time point in my experiments. The AMPK pathway was upregulated by liraglutide after 12 hours of HG exposure but this time, only 3 hours of 1nM of treatment was sufficient to lead to an increase in AMPK phosphorylation (Figure 9.11).

There is good evidence for the role of the AMPK pathway in mediating the anti-inflammatory effects of liraglutide in the endothelium. A key study was by Batchuluun and colleagues who used a model of human aortic endothelial cells exposed to 25mM glucose and demonstrated reactive oxygen species (ROS) production with dihydroethidium staining and fluorescent microscopy (Batchuluun et al. 2014). The cells were then treated with 10uM metformin and 30nM liraglutide either alone or in combination and the effects they had on the DAG-PKC-NADPH oxidase proteins assessed by plasmid transfection of PKC-**β** without a DAG-binding site, PKC translocation experiments, NADPH oxidase activity assays and Western blotting. The results showed that PKC translocation was stimulated by DAG (a direct by-product of glucose metabolism) preceding the detection of ROS, establishing a causal relationship. PKC**β** translocation and DAG levels, as measured by mass spectrometry, were reduced by metformin, liraglutide and combination of the 2 drugs. Since glucose incorporation into DAG has been previously shown to be inhibited by AMPK (Ido et al. 2002), the authors did further experiments with AICAR, an AMPK activator which confirmed that the effects of the drugs on PKC inhibition were mediated by AMPK and additionally had an effect on NADPH oxidase reduction. Similar to our results, they could not find further increase in AMPK phosphorylation with liraglutide 30nM compared to 3uM suggesting no dose-dependent relationship.

Animal studies (on diabetic rat myocytes) also support that theory that liraglutide exerts its anti-oxidative effects by modulating the AMPK-PKC-NADPH oxidase, via a mechanism independent of plasma glucose, insulin or body weight (Balteau et al. 2014; Inoue et al. 2015). The mechanism by which liraglutide stimulates AMPK is believed to be adenosine triphosphate (ATP)-dependent (since HG increases ATP production, which is an inhibitor of AMPK) (Carling et al. 2011) or ATP-independent (by increasing intracellular cyclic AMP (cAMP) levels) (Luo et al. 2013). cAMP and its downstream protein, protein kinase A (PKA), are key second messengers activated by the GLP-1 receptor, which directly inhibit NADPH oxidase and reduce oxidative stress in the renal vasculature (Hendarto et al. 2012). However AMPK was not specifically measured in this study so it was not possible to conclude if the anti-oxidant effect of liraglutide was due to cAMP increasing AMPK activity downstream, although that it is a plausible mechanism. Further proof of principle of the association between liraglutide and AMPK was provided by *Krasner et al*, who showed that the drug increased intracellular $Ca^{2+}$ concentrations, which in turn activated calmodulin-dependent protein kinase kinase-**β** (CaMKK**β**), a direct stimulator of AMPK phosphorylation (Krasner et al. 2014).

Aside from reversing DAG-PKC-NADPH oxidase signaling, liraglutide may also influence other downstream targets of AMPK to exert its anti-oxidant or anti-inflammatory effects, including suppression of NF-**κ**B and increasing NO production (Cacicedo et al. 2004).

More recently, a GLP-1 receptor/cAMP/PKA/liver kinase B1 (LKB1)/AMPK/eNOS cascade has been suggested for NO production by GLP-1 (Liu et al. 2012), but further experiments are needed to determine if this has any cross-talk with PI3K/Akt pathway for eNOS phosphorylation.

*Effect of Liraglutide on the Akt signaling pathway*

It is believed that activation of the GLP-1 receptors trigger at least 2 second messenger systems: 1) the cAMP-PKA pathway (which causes AMPK activation, as described above) and 2) indirect activation of epidermal growth factor activation leading to PI3K/Akt signaling (Buteau et al. 1999; Drucker 2006).

Native GLP-1 and GLP-1 agonists are effective in increasing the activity and phosphorylation of eNOS, which may be due to or independent of the GLP-1 receptor (Ding and Zhang 2012). This effect may be mediated by PKA, a major downstream protein of GLP-1 receptor stimulation action, that can directly phosphorylate eNOS at Ser1177 (Dong et al. 2013). The NO thus produced can protect endothelial cells by stimulating vasodilatation, scavenging ROS products and inhibiting leukocyte adhesion (Krasner et al. 2014; Ou et al. 2012).

Aside from a non-significant increase with 1nM liraglutide after 6 hour of treatment in the 48-hour time-point (which mirrored the effects seen with phospho-AMPK), I was not able to show any consistent suppression of eNOS phosphorylation after HG exposure or alleviation by liraglutide in my experiments. Possible reasons for this are technical issues with the antibody used or that these experimental conditions were not appropriate to show any changes in eNOS. Unfortunately, the study which was similar in design to ours which examined the effect of liraglutide in HG conditions did not evaluate phospho-eNOS expression or NO production (Batchuluun et al. 2014). Also, further experiments could be done to demonstrate decreased NO bioavailability aside from phospho-eNOS (Ser1177) protein expression, such as measurement of eNOS activity and eNOS mRNA levels.

Since a major upstream activator of eNOS activity is phospho-Akt (Dimmeler et al. 1999; Uruno et al. 2005), studying this protein can provide further insight into whether this signaling pathway is ameliorated by liraglutide in HG-mediated ROS.

Similar to the issue with phospho-eNOS, there have been no other studies which have examined phospho-Akt expression or regulation in experimental conditions similar to ours so no direct comparison is possible. However, it has been shown that exendin-4 (another GLP-1 analogue) leads to endothelial cell proliferation through upregulating phospho-Akt and phospho-eNOS expression (Erdogdu et al. 2010) and PKA was an important second messenger in this. Therefore the hypothesis is that another mechanism by which GLP-1 activates eNOS is through reversal of PKA/Akt inhibition. This may be particularly relevant in hyperglycaemic conditions since Akt phosphorylation has been shown to be downregulated by ROS activation, mediated by an increase in PTEN (Hou et al. 2015).

Therefore our study is potentially the first, to our knowledge, that suggests that liraglutide may exert its effect via the PI3K/Akt pathway under hyperglycaemic conditions, although the magnitude of effect is weak and may only be occur with acute exposure to HG.

Although *Dong et al* demonstrated PKA-mediated eNOS phosphorylation and increase NO levels with GLP-1 infusion, they did not observe any changes in Akt phosphorylation in skeletal muscle suggesting that this pathway may not be universally activated by GLP-1 (Dong et al. 2013). When evaluating oxidative-stress induced cell senescence in HUVECs, Oeseburg and colleagues concluded that the protective effect of GLP-1 was dependent on cAMP/PKA rather than PI3K/Akt (Oeseburg et al. 2010).

*Optimal dose and duration of treatment of Liraglutide*

There is no standard dose or duration of treatment of liraglutide for experiments on endothelial cells since different studies have had different methodologies and aims. In the *Batchuluun et al* study, the cells were only treated with 1 hour each of HG medium and liraglutide likely because they were doing several dynamic assays of

ROS detection, PKC translocation and so on; notably the cells were *pre-treated* with the drugs prior to HG exposure.

One hundred (100) nM liraglutide, which exceeds the therapeutic range obtained in patients taking the 1.8mcg/day preparation of liraglutide, was the maximum dose used in our study (Jacobsen et al. 2016). However, we also experimented with smaller doses of 1nM and 10 nM because: 1) there is no evidence regarding potential toxicity of very high doses of liraglutide and 2) anti-inflammatory and anti-oxidant effects of the drug have been found at concentrations of 30nM in cell models (Batchuluun et al. 2014; Shiraki et al. 2012).

In my study, one of the key questions initially was whether the ROS production increased with chronic exposure to HG which liraglutide may alleviate so a 6 to 10 day time course experiment was designed. However it transpired that cell apoptosis possibly due to senescence became apparent at durations beyond 4 days of HG, so we decided to focus on the 48hour time point and additionally studied a 12-hour time point as well. In accordance, the duration of liraglutide was also varied between 3 and 6 hours. Although not conclusive, it appears that longer exposure to HG requires a longer duration of liraglutide to observe a benefit possibly reflecting more oxidative stress-mediated damage that needs to be overcome.

*Translational / Therapeutic options*

In clinical settings, liraglutide treatment has been shown to improve endothelial function in patients with T2D, lending more support to the observations found at the tissue level. Various biochemical and physiological markers of endothelial dysfunction have been identified in clinical use and the effect of GLP-1 RAs on them studied. For example, liraglutide has been shown to reduce levels of urinary diacron-reactive oxidation metabolites (d-ROMs) in diabetic patients (Okada et al. 2014). Brachial artery FMD, measured by ultrasonography, represents endothelial NO

bioavailability and is widely accepted as an early marker of atherosclerosis (Corretti et al. 2002). However the effect GLP-1 RAs or liraglutide have on FMD is variable, possibly due to population differences (control of DM, pre-existing atherosclerosis, concomitant drugs) and the GLP-1 treatment used (Nomoto et al. 2015; Nystrom et al. 2004).

If further laboratory studies confirm the anti-oxidant / anti-inflammatory role of liraglutide on endothelial cell damage as suggested by my study and others, the next step would be clinical trials to identify a subgroup of diabetic patients who may benefit from GLP-1 RA treatment to reduce cardiovascular complications. For instance, 2 clinical studies on diabetic patients has shown that the endothelium becomes less sensitive to the protective effects of a GLP-1 infusion when hyperglycaemia is more pronounced (as measured by markers of oxidative stress and FMD) (Ceriello et al. 2011) and that the effect of exenatide on postprandial endothelial function was mediated by lowering of triglyceride levels (Koska et al. 2010).

*Strengths and weaknesses*

In this pilot study, I have shown through several cell culture and Western blot experiments a possible methodology for studying the mechanism by which liraglutide can alleviate oxidative stress in endothelial cells when exposed to HG conditions. Aside from Batchuluun et al, there have been no other studies, to our knowledge, which have studied the role of liraglutide in a hyperglycaemic milieu similar to what diabetic individuals would experience. Particularly, I was interested in examining the relative contributions of both the phospho-AKT and phospho-AMPK pathways, which have been separately shown to be important in alleviating oxidative stress. However this is a pilot study, which has a few important limitations. Firstly, in the absence of total-AKT and total-AMPK protein expression, the results from the phospho-isoforms must be interpreted with caution. This is possibly due to the protocol I used for reprobing, which meant that bands for the total isoforms did not show up clearly on repeat Western blots despite multiple attempts. These experiments

need to be replicated after optimisation of the stripping buffer and reprobing protocol. Additionally, further experiments need to be done to study gene expression of the relevant proteins (eg. by polymerase chain reaction) to confirm the findings of the Western blots. The generation and alleviation of oxidative stress under the various conditions used in the experiments can also demonstrated by dynamic fluorescence assays as proof of principle.

### 9.5. Conclusion

In the basic science part of my thesis, I have presented a methodology of studying the how liraglutide may alleviate oxidative stress in endothelial cells using cell culture and Western blot experiments. Putting the preliminary findings of these experiments and evidence from other studies together, my impression from this pilot study is that liraglutide inhibits ROS formation predominantly by the increasing AMPK phosphorylation, which results in decreased DAG synthesis and PKC activity, after acute and moderate-term exposure to HG. The downstream signaling pathways following the formation of ROS and endothelial cell damage are believed to include Bcl-2/BAX mediated cell apoptosis, which GLP-1 RAs may alleviate. The phospho-Akt pathway may also be activated by this drug, particularly with more acute exposure to HG and shorter duration of drug treatment. Therefore it is possible that liraglutide acting via AMPK may exert antioxidant/anti-inflammatory effects on endothelial cells with or without the PI3K/AKT/eNOS signaling pathway. If this observation is confirmed by further experiments, it could mean that newly diagnosed diabetic patients with acute hyperglycaemia-mediated endothelial dysfunction may benefit most from the cardiovascular protection of liraglutide.

# Chapter 10

# Overall discussion and conclusion

## 10.1. Introduction

Gestational diabetes mellitus (GDM) is a multifactorial heterogeneous condition which can have long-term consequences for the mother and her affected fetus(es). I have thus far described in my study how 2 potential biomarkers, namely vitamin B12 (B12) and glucagon-like peptide-1 (GLP-1), may be independently associated with this condition and also showed, at a tissue level, possible mechanisms by which GLP-1 agonist treatment may be beneficial in alleviating cell damage caused by hyperglycaemia.

The next logical step would be to examine if there are any treatment options (such as supplementation or fortification of B12 and GLP-1 agonist treatment respectively) that can be used in 'at-risk' pregnant women to reduce their metabolic risks. However prior to that, there are still a few issues which need careful consideration: the biomarkers I have studies are only associated with GDM, not causative, and one way to confirm these associations would be to evaluate other related factors with regards to GDM risk. Secondly, if impairments in B12 and GLP-1 are indeed linked to glucose intolerance in pregnancy, they should by definition resolve post-partum although it is unknown if there are any long-term 'metabolic memory effects'. Related to this, it is possible that the affected women may become pregnant again. This would put her and her future offspring at the same (if not higher) risk of GDM thereby making the inter-partum period an ideal time to intervene. Thirdly, there need to be more studies with regards to feasibility and safety of interventions such as B12 supplementation or GLP-1 potentiating treatment in pregnancy or post-partum. I will discuss these issues in turn, along with available evidence, in the following sections.

## 10.2. Risk of Type 2 diabetes and metabolic disease following GDM pregnancy

Gestational diabetes mellitus (GDM) resolves after delivery due to improvement of the insulin resistance induced by pregnancy and reduction in levels of diabetogenic hormones such as human placental lactogen, growth hormone and progesterone (C. Kim 2014). Women with GDM are advised to have post-natal follow-up which varies in different countries and areas based on local practice, but by and large includes a fasting plasma glucose, annual HbA1c and rarely a 75g glucose tolerance test (National Institute for Health and Care Excellence 2015a). Longitudinal studies have

shown that women with a history of a GDM pregnancy have a 40% chance of developing it in a subsequent pregnancy, compared to around 4% risk in the background population (Ehrlich et al. 2011; Getahun et al. 2008). These women also have a higher risk of T2D as early as 9 months after the index pregnancy and a meta-analysis found a 7-fold increase risk over a follow-up time of 2 - 25 years (Bellamy et al. 2009; Feig et al. 2008). In addition, I discussed in Chapter 9 how these women are also at increased risk of cardiovascular events, macro- and micro-vascular disease, mediated in part by endothelial dysfunction (Carr et al. 2006; C. Kim 2014; Retnakaran and Shah 2017).

Strategies which are advocated to reduce a woman's intrapartum and future risk of diabetes include weight loss, particularly for women who were overweight or obese in the index pregnancy, intensive lifestyle modification (as shown in the Diabetes Prevention Program) and breastfeeding (Ehrlich et al. 2011; O'Reilly et al. 2011; Ratner et al. 2008; Ziegler et al. 2012).

I would argue that in addition to these generalised measures, it is important to consider novel factors (e.g. B12 insufficiency or impaired early GLP-1 response to glucose load) that may have put her at risk of GDM in the index pregnancy and therefore future GDM in a following pregnancy or T2D. If any association is indeed proven between the factors I have studied and metabolic health outcomes, accurate risk stratification of the affected women should involve re-evaluation of these risk factors post-pregnancy and correction, where appropriate. I will discuss possible strategies of doing this in the following sections.

### 10.3. Confirming association and establishing causation between vitamin B12 and GDM / related metabolic outcomes

In Chapter 5, I have shown that low maternal B12 insufficiency is linked to higher odds of obesity, GDM and additionally may have an independent effect on fetal macrosomia. The possible mechanisms associated with this risk have been discussed and are believed to include adipocyte dysfunction, abnormal lipid metabolism and hypomethylation of cholesterol biosynthesis pathways.

However, the one-carbon metabolism cycle at a cellular level is complex with several interdependent pathways and if B12 is indeed causative for any metabolic insults to pregnant women or their offspring, derangements in other related biomarkers are to be expected too. It should be stressed that to confirm any association and establish potential causality between B12 or other related biomarkers with GDM, they should be measured pre-conceptionally or in early pregnancy and such studies are lacking at present but are ongoing (e.g. PRiDE study in University of Warwick).

In our study, we found no significant relationship between maternal folate levels and obesity or GDM (Chapter 5) but surprisingly, folate showed a positive relationship with the risk of fetal macrosomia (i.e. highest risk in women with folate in the highest quartile, OR 4.99, adjusted p=0.02). Taken in the context of an inverse relationship between maternal B12 and risk of macrosomia of the same magnitude (OR 5.3 for macrosomia in the lowest quartile of B12, p=0.05), we can speculate that the combination of low B12 and high folate may contribute to excessive fetal growth, potentially due to exposure to a hyperglycaemic intrauterine environment. Interestingly, in the Pune Maternal Nutrition Study, children born to mothers with B12/folate imbalance in the 3$^{rd}$ trimester of pregnancy were observed to be more centrally adipose and insulin resistance at age 6, although this is a very different population with different inherent risks (C. S. Yajnik et al. 2008).

There is no convincing evidence of an inverse relationship between folate and risk of GDM or obesity, although it does predict lower fetal birthweight (BW) (Bergen et al. 2012; Relton et al. 2005).

Homocysteine has not been independently linked to maternal obesity or GDM but in a meta-analysis of over 21000 individuals, was associated with a small but significant risk of small for gestational age (SGA) births (OR 1.25 95% CI 1.09, 1.44, 1SD increase in Hcy associated with 31g increase in BW) (Hogeveen et al. 2012). *Yajnik et al* proved a causal relationship between hyperhomocysteinaemia and low BW using instrumental variable analysis of a polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene (C. S. Yajnik et al. 2014). Hyperhomocysteinaemia among pregnant women has been attributed to both B12 and folate insufficiencies depending

on the population studied and their micronutrient status (Bondevik et al. 2001; Selhub et al. 2007a; C. S. Yajnik et al. 2014).

An exploratory metabolomics study, with 29 GDM women and 25 controls has identified that urinary choline and associated plasma betaine excretion was higher in the former (Diaz et al. 2011). Choline metabolism has been shown to be altered in hyperhomocysteinaemia since the Hcy to methionine conversion is coupled with betaine metabolism and choline is a direct precursor of betaine. Aside from choline, there are several other markers relevant to the B12-folate-Hcy-methionine cycle, such as AdoMet, AdoHcy, vitamin B6 and cysteine, which remain to be studied with regards their associations to metabolic disease in pregnancy. These can be investigated via a metabolomics approach or other exploratory studies to begin with before being tested in clinical samples.

### 10.4. Supplementation (or fortification) of vitamin B12

From Chapter 4 and 5 of my study, is apparent that B12 insufficiency during pregnancy is prevalent in several populations across the world and may potentially be associated with adverse consequences for mother and baby (e.g. GDM, high BMI, extremities of birthweight). However to strengthen these observations and prove causality, it would be necessary to show, via a randomised control trial, that B12 supplementation to mothers preconceptionally or during pregnancy can reverse these outcomes. Prior to that, it is necessary to conduct small feasibility trials to determine the optimal route, dose or duration of B12 which should be given to pregnant women with B12 insufficiency. There also needs to be careful consideration about the primary outcome which is being studied as that will influence the design and conduct of the trial. Possibilities include 1) normalisation of serum B12 levels, Hcy or MMA levels, 2) prevention of GDM or other associated metabolic outcomes and 3) prevention of extremities of fetal birthweight or prematurity

There have no trials so far where offspring BW has been reported a primary outcome in pregnant women supplemented with oral B12 alone versus placebo. An placebo-controlled trial of B12 supplementation for pregnant women from 14 weeks of

pregnancy found higher maternal and infant plasma B12, folate and lower Hcy and MMA concentrations in the treated group but no significant reduction in the rate of fetal growth retardation (secondary outcome) (Duggan et al. 2014). A meta-analysis of multiple micronutrient supplementation trials (which typically contains 1 RDA of B12), done in 12 low-income countries in Asia, Africa and Central/South America, showed that supplementation was associated with modest increase in BW (effect size +22g, p=0.002), reduced low birthweight (LBW), SGA and increased large-for-gestational age babies, all of which were significant (Fall et al. 2009). Surprisingly, the effect was larger as maternal BMI increased with no or even a negative effect in mothers with BMI <20kg/$m^2$.

The more recently conducted Mumbai Maternal Nutrition Project, showed no overall increase in neonatal BW in the supplemented group but subgroup analysis on mothers who had the supplements for more than 3 months before conception (per protocol analysis), found that there was a positive effect on BW, particularly as maternal BMI increased (overall effect size +48g (p=0.046); maternal BMI >21: +113g) (Potdar et al. 2014). This suggests that optimisation of maternal B12 in the periconceptional period may be more beneficial than during pregnancy itself. The available data support the hypothesis that even in populations which show an apparent association between low B12 levels and LBW offspring, the most 'at-risk' (i.e. underweight) women are unlikely to benefit from micronutrient supplementation alone as their nourishment and macronutrient status needs to be optimised first.

Another reason for the disparity observed between populations who benefit from supplementation and those who do not, may be the baseline micronutrient status of the women. For example, in the trial which showed 64g increase in BW, the Nepalese women had relatively a higher rate of folate deficiency (11%) and lower rate of B12 deficiency (27%) (Christian et al. 2003; Stewart et al. 2011). In comparison, in the Mumbai trial there were no micronutrient levels reported but the corresponding folate and B12 and folate insufficiency rates in pregnant women were from India are around <5% and 40-60% respectively (Krishnaveni et al. 2014; C. S. Yajnik et al. 2008), suggesting that the combination of low B12 and high folate may be more deleterious in the pregnant women and their offspring, which may not be so easily reversed with micronutrient supplementation.

It can be concluded from the above evidence and my findings from Chapter 4 and 5 that B12 supplementation with the aim for increasing BW or reducing LBW / SGA are probably not necessary and are unlikely to be effective in the context of the UK population due to low inherent risk. However low B12 status is still an important problem in our population (26% of high-risk pregnant women in our population and 12% of women of child-bearing age nationally have levels <150pmol/l, which in the latter group was associated with hyperhomocysteinaemia). Therefore is there a role for B12 supplementation to reduce GDM outcomes in pregnant women with certain risk factors (e.g. obesity, vegetarianism) or should national policies be reviewed with regards to increasing the recommended nutrient intake (RNI) for B12, targeting women of child-bearing age? Paucity of randomised control trials (RCT) on B12 supplementation in pregnancy or pre-conception makes it difficult to answer this question but more robust evidence and feasibility studies are needed first. Some of these issues that need to be determined are the optimal route and dose of B12 supplementation.

*Route*

The 2 forms of B12 commonly used in treatment are cyanocobalamin (oral route) or hydroxocobalamin (intramuscular route). As alluded to in Chapter 7, B12 in animal products is predominantly protein-bound while in fortified foods or vitamin supplements it is in the free form. B12 in food exists as hydroxocobalamin while in vitamin supplements it is cyanocobalamin, which was believed to be more easily digested in the gastrointestinal tract than hydroxocobalamin anecdotally. However a recent animal study has shown comparable absorption rates (Kornerup et al. 2016).

It is currently not standard practice in the UK, to give oral B12 supplements to pregnant women at risk of, or diagnosed, with B12 insufficiency. If a woman is found to have low B12 levels either in the pre-conceptional or antenatal period which is thought to be due to dietary deficiency, the practice in our NHS Trust is to give a 3 loading doses of 1mg vitamin B12 intramuscular injection and repeat the B12 levels. If the insufficiency is a chronic problem, general practitioners will usually prescribe 3-monthly B12 injections as maintenance. Such practice is broadly in keeping with the NICE clinical knowledge summary with the added recommendation of giving 50 –

150mcg oral cyanocobalamin daily for younger people or non-vegans (National Institute for Health and Care Excellence 2015b)

*Dose and risks of over supplementation*

Although the RNI for B12 intake in adults in the UK is 1.5mcg/day, commercial oral formulations contain anywhere between 2.5 – 100mcg (e.g. Pregnacare® contains 6mcg, Boots® Pregnancy Support tablets contain 2.5mcg, Holland and Barrett® B12 Tablets contain 100mcg). One rationale for taking higher doses is that bioavailability of B12 decreases to about 5-10% with higher concentrations, similar to dietary intake.

High B12 intakes and blood levels are generally considered safe but no studies have specifically examined the adverse effects over a prolonged period, especially during the periconceptional stage. While most of the excess amounts of water- soluble vitamins are excreted in urine, urinary excretion of B12 does not increase when large doses of B12 are supplemented (Fukuwatari et al. 2009). It is believed that excess amounts are stored in the liver and the effect of this especially during pregnancy is unknown. B12 crosses the placenta, probably through active transport as cord blood levels are higher than maternal level (Chapter 4).

Therefore whilst B12 supplementation may an appropriate and effective treatment in pregnant women diagnosed with insufficiency, there is still insufficient evidence with regards to implanting this on a population level. An alternative is to consider co-fortification of B12 together with folate which is timely given the on-going discussions which are taking place in the UK. Some of the challenges with B12 fortification (in contrast to folate) are reduced bioavailability when attached to certain foods, saturation of intrinsic-factor process at high intakes and the effect of co-existing malabsorptive conditions (Carmel 2008).

In conclusion, there is still insufficient evidence to recommend routine B12 supplementation in pregnant women or fortification on a population level. However based on my findings from Chapter 6, more consideration could be given to increasing the RNI of B12 intake to al least match European recommendations of 4.5mcg/day particularly in women of reproductive age. This, accompanied by more

public health messages to raise the awareness about the importance of adequate B12 status for women planning pregnancy, may mean that they enter pregnancy with higher B12 reserves.

### 10.5. Biomarkers related to GLP-1

From my results in Chapter 8 and surrounding discussion, it is likely that there is association between GLP-1 levels (particularly in the early phase after an oral load), impaired insulin secretion and GDM. However the situation with the other incretin hormones is less conclusive.

Glucose-dependent insulinotropic polypeptide (GIP) is the other major incretin hormone secreted by the K-cells of the distal small intestine in response to oral load and in addition to potentiation of insulin secretion, has systemic effects which are quite diametrically opposite GLP-1. It has been shown to have diabetogenic/adipogenic properties which could mediate the link between high fat diets and metabolic outcomes such as obesity, insulin resistance and T2D (Chia et al. 2009; Irwin and Flatt 2009) There does not seem to be a consistent change in GIP levels in GDM with some studies one study showing no change (Cypryk et al. 2007) and one showing increase in fasting GIP concentrations only compared to controls (Bonde et al. 2013).

Interestingly, it has been suggested that obese individuals, such as those with GDM or a history of it, have a greater insulinotropic in response to GIP administration under experimental conditions, which may be a compensatory mechanism to their insulin resistance (Meier et al. 2005). This is at variance with the reduced insulinotropic response to GIP observed in type 2 diabetics and their first-degree relatives, which was thought to represent a genetic defect in GIP action in predisposed individuals (Meier et al. 2001; Nauck et al. 1993). Therefore further studies are needed to determine any association between GIP secretion or action and glucose intolerance in pregnancy.

Anyhow, latest evidence from non-pregnant adults is that that GIP and GLP-1 responses are independent of each other and are regulated by different factors (Vollmer et al. 2008). Some predictors of reduced GLP-1 response during a GTT in

non-pregnant adults, which warrant further study in GDM, are fasting glucagonaemia, impaired glucagon suppression during GTT and lower fasting free fatty acid concentrations. GLP-1 action, but not GIP, has been found to contribute to pancreatic B-cell growth and increased intrapancreatic insulin content in pregnant mice providing further proof of principle that the former hormone is required for increased insulin production to compensate for the insulin resistance of pregnancy (Moffett et al. 2014).

### 10.6. GLP-1 levels and incretin response following GDM pregnancy

There is good evidence that any impairment in GLP-1 response during a GDM pregnancy improves post-natally if normal glucose tolerance is established but does not normalise entirely. Two of the 5 studies which measured GLP-1 levels during pregnancy did follow up studies with a repeat glucose challenge post-partum (summarised in Table 7.1) (Bonde et al. 2013; Lencioni et al. 2011). Bonde and colleagues found that the total GLP-1 response in GDM women increased by over 50% compared to their pregnancy levels (8.4 vs. 5.5 nM x min, p=0.005) (Bonde et al. 2013). These women appear to have persistent abnormalities in their GLP-1 levels compared to their counterparts who did not have GDM, with reduction in fasting GLP-1 levels and total GLP-1 response. In fact, *Lencioni et al* found that when the GLP-1 levels were corrected for prevailing glycaemia, the GLP-1/glycaemia ratio showed an almost flat line indicating that previous GDM women continue to mount an inadequate incretin response. However other cross-sectional studies do not support an impairment of GLP-1 secretion in women with a history of GDM (Meier et al. 2005).

It has been shown that impairment of GLP-1 response may be an early insult in the pathogenesis of T2D as shown in experiments on previously healthy individuals who had insulin resistance induced by steroid therapy (discussed in Chapter 7). Beta-cell secretory capacity was normal in these individuals as observed by the insulin secretion in response to an intravenous glucose load but that in response to an oral load was specifically reduced implicating defective incretion hormone secretion/action.

Similar to the impaired GLP-1 response observed in previously healthy individuals who had insulin resistance induced by steroid therapy (discussed in Chapter 7), my study has shown that the women who are predisposed to develop diabetes in later life may show early abnormalities in their incretin response (including GLP-1 levels) and this manifests in pregnancy as GDM when their beta-cell capacity cannot increase sufficiently to cope with physiological insulin resistance. It will be insightful to have long-term follow up studies on such women with previous GDM and impaired GLP-1 levels to look specifically at factors such as whether their time to development of T2D was shorter or if they presented with more complications when compared to previous GDM women without impaired GLP-1 levels. If so, it could be possible to identify a subgroup of women with previous GDM for more targeted follow up and perhaps early intervention to prevent future T2D.

### 10.7. GLP-1 axis potentiating drugs in pregnancy and post-partum

GLP-1 receptor agonists (GLP-1 RA) are currently not recommended during pregnancy because animal studies have shown that Liraglutide is associated with early embryonic deaths and reduced fetal growth in mid-gestation (Novo Nordisk Limited 2016).

However, the post-partum period may be an ideal time to intervene in women who have had a GDM pregnancy given their high risk of progression to T2D. Drugs that modulate the entero-insular axis will be even more favourable if defects in GLP-1 secretion are confirmed to be account for decreased first-phase insulin secretion or impaired incretin response in GDM or early T2D. Indeed, exenatide (another GLP-1 RA) has been associated with increase in first and second phase insulin secretion although an intravenous glucose load was used for this experiment (Fehse et al. 2005).

Two RCTs are currently being carried out of liraglutide vs. placebo and liraglutide/metformin vs. placebo/metformin in women with a diagnosis of GDM during pregnancy within the last 1-10 years (Elkind-Hirsch 2016; Foghsgaard et al. 2013). The estimated total sample size is 100 and 150 respectively and the open label observation phase is expected to be complete in 2019/2020 with results available shortly after. It will be interesting to note if, in addition to the primary outcomes of glucose tolerance and insulin sensitivity markers, the investigators report any changes

in markers of vascular complications in these patients which would tie in with the anti-oxidant effects of Liraglutide observed in Chapter 9.

There is no data on the safety of any other drugs which could potentiate incretin hormones (e.g. DPP-4 inhibitors) during pregnancy and their use is not recommended. However metformin, which is widely used in the management of GDM, has been shown in lean and obese non-pregnant women with polycystic ovarian syndrome to increase GLP-1 secretion after a GTT (Svendsen et al. 2009). The positive effect metformin has on GLP-1 levels is a direct one (i.e. not only related to weight loss or improvement of insulin resistance) and is believed to be due to enhancing GLP-1 biosynthesis and secretion as well as inhibiting the degradation enzyme dipeptidyl-4 (B. D. Green et al. 2006; Lindsay et al. 2005).

While long-term use of these medications in women of reproductive age are not desirable, they may be useful as an adjunct approach in certain individuals if efficacy and safety are proven in RCTs.

### 10.8. 'Individualised' management of GDM

It can be seen from the earlier results and discussions that while the burden of GDM is increasing rapidly, it continues to remain an incompletely understood condition with a heterogeneous pathophysiology. This can, in turn, have differential adverse effects on both the affected woman and her offspring. Therefore novel biomarkers may help to better diagnose and characterise these women with the ultimate aim of tailoring their management in pregnancy and in the prevention of future metabolic disease.

For instance through Chapters 4 to 5, I have shown that in addition to GDM, low B12 is independently associated with higher BMI and extremities of fetal birthweight and mechanistic studies suggest that the process of adipocyte dysfunction and insulin resistance may mediate this link. My study of GLP-1 levels in Chapter 7 has found that lower GLP-1 levels early in a glucose tolerance test are independently related post-glucose challenge hyperglycaemia and there is good evidence that this could be due to impaired beta-cell function, which itself may progress post-pregnancy.

Other non-pharmacological strategies which have been tried, but not definitely shown to be beneficial in the management of GDM are low-glycaemic index or carbohydrate diets (Hernandez et al. 2016; Markovic et al. 2016) and encouraging breaks in sedentary behaviour to reduce post-prandial glucose (Henson et al. 2013).

Therefore the emerging theme is that there can be multiple mechanisms explaining the metabolic insults which give rise to glucose intolerance in pregnancy (e.g. predominant insulin resistance or insulin secretion or combination of factors) and different biomarkers can provide further insight into these. Current practice dictates that all women with GDM are managed along similar lines (blood glucose monitoring, oral hypoglycaemic agents or insulin and fetal growth monitoring as outlined according to NICE guidelines) (National Institute for Health and Care Excellence 2015a). However, I argue that a "one size fits all" approach cannot be applied to all women with GDM and more careful consideration needs to be given to her individual risk factors, aided by identified and yet-to-be identified biomarkers, which can enable a more focussed and targeted management to be provided both during pregnancy and afterwards.

## 10.9. Fetal programming of metabolic disease

The other problem with current management strategies for GDM is that diagnosis and treatment typically begins in the early 3$^{rd}$ trimester providing a window of only 10 to 12 weeks for intervention prior to delivery. Exposure of the fetus to hyperglycaemia prior to adequate control of GDM as well as the effects of in utero exposure to other insults which have predisposed the pregnant woman to GDM, may result in long-term consequences for the offspring extending to adulthood. Longitudinal cohort studies have shown that offspring of diabetic mothers (GDM or type 1 diabetes), mediated in part by their own body mass indices, are prone to obesity, insulin resistance and other abnormalities from childhood thereby perpetuating the trans-generational effect of metabolic disease (Boney et al. 2005; Clausen et al. 2008; Clausen et al. 2009).

In the course of my research, I have found that low maternal B12 levels is associated with small for gestational age babies in women from high-risk populations (Chapter

1) and in our population, was linked to over 5-times higher risk of fetal macrosomia in a subgroup of non-diabetic women. Fetal birthweight might only be one of the mechanisms by which B12 can influence offspring metabolic health and birthweight itself may be a surrogate marker for other epigenetic, nutritional or metabolic processes linked to micronutrient insufficiency. More longitudinal cohort and mechanistic studies are urgently needed to explore any possible between B12 and other 1-carbon metabolite status in pregnancy and long-term maternal and child health.

Although I did not examine any fetal outcomes with regards to impaired GLP-1 concentrations or response in my study, this is something which can be studied in the cohort of patients I recruited who have had detailed anthropometric measurements at birth as part of the PRiDE study. One study has shown that lower fasting GLP-1 levels were correlated with higher fetal abdominal circumference and birthweight in non-overweight/obese, non-diabetic pregnant women (Valsamakis et al. 2010). Interestingly the GLP-1 impairment was also linked negatively to parameters of insulin resistance, which may of course mediate the higher fetal anthropometry.

The transgenerational perpetuation of diabetes may also involve the GLP-1/insulin axis as observed in long-term follow-up studies. Non-diabetic normal BMI offspring of diabetic mothers (both T1D and GDM) demonstrate impaired fasting GLP-1 levels, total GLP-1 response and glucagon suppression after an oral challenge in childhood and early adulthood compared to offspring not exposed to hyperglycaemia in utero (Chandler-Laney et al. 2014; Kelstrup et al. 2015). In the absence of incretin hormone measurements in the pregnant women during the index pregnancy and correlation analyses, it is not possible to determine if these effects are due to genetic impairments of incretin hormone secretion/action or if they represent an early tendency to diabetes and metabolic disease due to altered in utero programming but the inclusion of offspring of T1D women in 1 study supports the latter theory.

The ideal biomarkers should therefore enable early identification of women at risk of GDM not only for the benefit of improving their glycaemic control during pregnancy, but also to minimise the exposure of their fetuses to metabolic insults which could programme them for chronic diseases later on.

## 10.10. Conclusion

In summary, my study supports the hypothesis that GDM is a multifactorial heterogeneous condition that is still incompletely understood. The 2 novel biomarkers I have studied, namely vitamin B12 and GLP-1, provide useful insight into different pathogenic mechanisms which exist in diabetes in pregnancy. Specifically, B12 may be related to obesity, GDM and birthweight abnormalities while impaired GLP-1 secretion (particularly in the early phase following a glucose challenge) may predict impaired beta-cell function and postprandial glucose. These observations warrant replication in larger prospective studies. If the associations are proven and potential causality identified, these biomarkers can be used to risk stratify these women, both during the pregnancy and afterwards, to reduce her likelihood of future GDM, T2D and related complications. Therefore, adopting a more 'individualised' approach to manage this escalating problem can positively influence the health of young women and their offspring to tackle the global epidemic of diabetes.

## Appendix 1 – Supplemental Tables (Chapter 4)

### 1.1 Quality assessment criteria used to grade studies[1]

| Question no | Criteria | Comment |
|---|---|---|
| 1 | The study addresses an appropriate and clearly focused question. | |
| **Selection of subjects** | | |
| 2 | The two groups being studied are selected from source populations that are comparable in all respects other than the factor under investigation. | |
| 3 | The study indicates how many of the people asked to take part did so, in each of the groups being studied. | |
| 4 | The likelihood that some eligible subjects might have the outcome at the time of enrolment is assessed and taken into account in the analysis. | |
| 5 | What percentage of individuals or clusters recruited into each arm of the study dropped out before the study was completed. | Not assessed for some cross-sectional studies |
| 6 | Comparison is made between full participants and those lost to follow up, by exposure status. | Not assessed for some cross-sectional studies |
| **Assessment** | | |
| 7 | The outcomes are clearly defined. | |
| 8 | The assessment of outcome is made blind to exposure status. | |
| 9 | Where blinding was not possible, there is some recognition that knowledge of exposure status could have influenced the assessment of outcome. | |
| 10 | The measure of assessment of exposure is reliable. | |
| 11 | The measure of assessment of exposure is valid. | |

| | | |
|---|---|---|
| 12 | Exposure level or prognostic factor is assessed more than once. | |
| **Confounding** | | |
| 13 | The main potential confounders are identified and taken into account in the design and analysis. | |
| **Statistical analysis** | | |
| 14 | Confidence intervals are provided | |

[1]Adapted from Scottish Intercollegiate Guidelines Network (SIGN)

## 1.2 Quality assessment of studies included in the 'Prevalence of B12 insufficiency' sub-review

| Study Reference | Overall study quality grade |
|---|---|
| Whiteside, 1968 | - |
| Roberts, 1973 | + |
| Jiang, 2005 | ++ |
| Murphy, 2007 | ++ |
| Garcia-Casal, 2005 | + |
| Ray, 2008 | + |
| Kosus, 2012 | ++ |
| Dwarkanath, 2013 | ++ |
| Heppe, 2013 | ++ |
| Samuel, 2013 | ++ |
| Shamim, 2013 | ++ |
| Shields, 2011 | + |
| Lowenstein, 1960 | - |
| Jacob, 1976 | + |
| Yajnik, 2008 | ++ |
| Katre, 2010 | + |
| Marzan, 1971 | + |
| Areekul, 1976 | + |
| Knight, 1991 | + |
| Bruinse, 1995 | + |
| Ackurt, 1995 | ++ |
| Pagan, 2002 | + |
| Park, 2004 | ++ |
| Li, 2008 | ++ |
| Takimoto, 2007 | ++ |
| Goedhart, 2011 | ++ |
| House, 2000 | - |
| Milman, 2006 | ++ |
| Wu, 2013 | ++ |
| Hinderaker, 2002 | ++ |
| Zachau-Christiansen, 1962 | - |
| Yusufji, 1973 | + |
| Baker, 1975 | + |
| Osifo, 1976 | - |
| Bjorke-Monsen, 2001 | ++ |
| Pathak, 2007 | + |
| Krishnaveni, 2009 | ++ |
| Cole, 1974 | + |
| Colman, 1975 | - |
| Frery, 1992 | ++ |
| Giugliani, 1984 | + |
| Ho, 1987 | + |
| Black, 1994 | + |
| Ma, 2004 | + |
| Hall, 2007 | ++ |
| Gibson, 2008 | + |
| Schulpis, 2004 | + |
| Koc, 2006 | + |
| Barbosa, 2008 | ++ |
| Hussein, 2009 | + |
| Vanderjagt, 2009 | + |
| Halicioglu, 2012 | + |

| | |
|---|---|
| Balci, 2014 | + |
| Guerra-Shinoara, 2004 | + |
| Abdelrahim, 2009 | ++ |
| Cook, 1971 | ++ |
| Jacquemyn, 2014 | + |

Table showing the methodological quality assessment of studies included in the 'Prevalence of B12 insufficiency' sub-review based on the checklist for cohort studies adapted from SIGN. The studies are listed in order in which they are presented in Table 1.

++: 'All or most of the criteria fulfilled' (i.e. ≥ 67% of criteria fulfilled), +: 'Some of the criteria fulfilled' (i.e. 34-66% of criteria fulfilled),

-:'Few or none of the criteria fulfilled' (≤ 33% of criteria fulfilled)

### 1.3 Quality assessment of studies included in the 'B12 insufficiency and birthweight' sub-review

| **Study Reference** | **Overall study quality grade** |
|---|---|
| Muthayya, 2006 | ++ |
| Dwarkanath, 2013 | ++ |
| Furness, 2013 | ++ |
| Hogeveen, 2010 | ++ |
| Sukla, 2013 | + |
| Ubeda, 2011 | + |
| McGarry, 1972 | - |
| Krishnaveni, 2014 | ++ |
| Baker, 1977 | - |
| Navarro, 1984 | - |
| Abbas, 1994 | + |
| Lindblad, 2005 | ++ |
| Yajnik, 2005 | ++ |
| Mamabolo, 2006 | + |
| Abraham, 2013 | + |
| Gomes, 2010 | + |
| Hay, 2010 | + |
| Relton, 2005 | + |
| Takimoto, 2007 | ++ |
| Pagan, 2002 | + |
| Faintuch, 2009 | - |
| Frery, 1992 | ++ |
| Guerra-Shinoara, 2004 | + |

Table showing the methodological quality assessment of studies included in the 'B12 and birthweight' sub-review based on the checklist for cohort studies adapted from SIGN. The studies are listed in order in which they are presented in Tables 4.3-4.5.

++: 'All or most of the criteria fulfilled' (i.e. ≥ 67% of criteria fulfilled), +: 'Some of the criteria fulfilled' (i.e. 34-66% of criteria fulfilled),

-:'Few or none of the criteria fulfilled' (≤ 33% of criteria fulfilled)

## Appendix 2 – Supplementary Table (Chapter 5)

### A2.1 Table of characteristics of no-GDM women who did and did not undergo GTT

| Variables | GTT | No GTT |
|---|---|---|
| Number (%) | 90 (44.8) | 111 (55.2) |
| Age (yrs) | 30.2 ± 5.9 | 29.1 ± 5.8 |
| BMI ($kg/m^2$) § | 29.7 ± 8.5 | 24.3 ± 4.5 ***[a] |
| Obesity (%) | 43.3 | 4.5 *** |
| Current smokers (%) | 21.1 | 18.9 |
| Parity | 1.0 ± 1.1 | 1.1 + 1.2 |
| Ethnicity (%) | | |
| European | 87.8 | 87.4 |
| South Asian | 8.9 | 7.2 |
| Afro-Caribbean | 1.1 | 1.8 |
| Other | 1.1 | 0.9 |
| Gestation of B12 bloods (weeks) | 26.5 ± 5.7 | 25.9 ± 5.8 |
| Vitamin B12 (pmol/l) § | 180.4 (146.7, 221.6) | 218.4 (163.1, 259.8) * |
| Vitamin B12 deficiency (<150pmol/l) (%) | 25.6 | 18.9 |
| Serum folate (nmol/L) § | 22.0 (14.5, 34.9) | 19.7 (14.3, 34.0) |
| Serum folate deficiency (<7nmol/l) (%) | 1.1 | 0.9 |

Continuous variables are mean ± SD (or median (IQR)), categorical variables are percentages

a: p-value as compared to undergone-GTT group, *p<0.05, ***p<0.001

§: Log-transformed for statistical comparison

## Appendix 3 – Study documents for PRiDE-GLP1 clinical study (Chapter 8)

### A3.1 Participant Information Sheet

Warwick Medical School

PRiDE MAKING YOUR PREGNANCY COUNT

**Participant Information Sheet**

**Study Title: Role of GLP-1 Levels in gestational diabetes mellitus (PRiDE-GLP1 Sub-study)**

You are being invited to take part in a clinical study to look at whether a hormone known as Glucagon-Like Peptide 1 (GLP-1) is altered in mothers who are diagnosed with diabetes in pregnancy. This is a small addition to the PRiDE study, which you are already a part of. However, before you decide whether or not you want to take part in this sub-study, you should understand what it involves. Please take time to read this information sheet and discuss it with family and friends if you wish.

**What is the purpose of the study?**

GLP-1 is a hormone released by the gut in response to food. It helps our pancreas to produce more insulin which in turn helps to the lower blood sugar levels after a meal. Studies have shown that GLP-1 levels are reduced by up to 50% in adults with Type 2 Diabetes (T2D) and drugs which mimic the action of GLP-1 are routinely used to treat diabetes. It has also been shown that in people at higher risk of T2D, those who have defective GLP-1 secretion are more likely to develop full-blown T2D. Gestational diabetes mellitus (GDM) is considered to be a T2D-like state brought on by pregnancy. Though it resolves in most mothers after delivery, mothers with history of GDM have 7-8 times higher risk of T2D later on in life. There is little evidence on whether GLP-1 levels are faulty in GDM. This study is therefore designed to look at whether GLP-1 levels are indeed faulty in mothers who develop GDM. If it is, it provides a novel opportunity for improving the way GDM mothers are treated both during pregnancy and after delivery to reduce their likelihood of developing T2D in the future.

**Why have I been chosen?**

You have been chosen because you are currently in the PRiDE Study and are due to have your glucose tolerance test (GTT) soon.

**Do I have to take part?**

Participation in this sub-study is entirely voluntary. If you decide not to participate, you can still carry on in the main PRiDE Study and the standard of your antenatal care will **not be affected** in any way.

**What will happen to me if I take part?**

We will ask you to sign a consent form to confirm your willingness to participate. This sub-study only involves additional blood tests during your GTT**.**

To accurately determine how GLP-1 levels change in response to consuming glucose, it is necessary to check the GLP-1 levels every 30 mins during your 2-hour GTT. A cannula will be inserted into a vein in your forearm at the start of your GTT and blood will be drawn at 0,

30, 60, 90, 120 min. Normal GTT bloods are drawn only at 0 and 120 min. While the regular GTT involves 2 separate needles, the additional samples for this sub-study only involves the initial insertion of the cannula. Around 10 mls of extra blood will be taken for this sub-study at each time point.

If you are diagnosed to have GDM, you will be offered a GTT around 6 weeks after delivery as part of your regular clinical care. For this GLP-1 sub-study, we would require the 3 additional blood samples as described above to test the GLP-1 levels during this GTT too.

**What are the possible advantages and disadvantages of taking part?**

The results of this study will help to determine if reduced GLP-1 levels is a factor contributing to GDM and diabetes risk in mothers after their pregnancy. If so, it can pave way for future novel treatment of mothers with GDM either during or after their pregnancy. This may be relevant for you directly if you are planning any future pregnancies too. There are no specific disadvantages apart from insertion of a small cannula in your forearm for 2 hours and the additional blood samples described above. However, this will only involve 1 needle insertion as opposed to the 2 for your normal GTT.

**What will happen if I change my mind about taking part?**

You are free to withdraw from the study at any time, without giving a reason if you do not wish to. All the blood samples you have provided us with until that point can be destroyed if you do not want them to be used. The remainder of your and your baby's care will continue as normal.

**Will my taking part in this study be kept confidential?**

Your participation in the study will be kept entirely confidential. Any personally identifiable information will be stored in a password-protected database or secure area that only the research staff and study regulatory authorities will have access to. You will be allocated a unique study number and any samples we take from you will only be identified by this number when processed or stored in the lab.

**Who has funded and reviewed this study?**

The study is being funded by West Midlands (South) Comprehensive Local Research Network after thorough review. The NHS Research Ethics Committee has approved the study. Your local NHS Trust and antenatal department have also approved it.

**What if there is a problem?**

Your rights to the regular NHS complaints procedure are not affected in anyway by taking part in this study. If you are concerned about any aspect of the study or unhappy with how you were treated by the research staff at any point, please contact Ms Nicola Owen, University of Warwick Research Support Services (Tel: 024 7652 2785, email: Nicola.Owen@warwick.ac.uk). You can contact Dr P Saravanan or his research team on 02476153592 if you would like more information about the study. If you would like to speak to someone independent about taking part in this study please contact the Patient Advice and Liaison Service on 02476865550.

**What will happen to the results of this study?**

You will be informed of the summary of the results, after all the data analyses are complete. The scientific results will be published in medical journals and presented in conferences.

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## A3.2 Consent form

Warwick Medical School

PRiDE MAKING YOUR PREGNANCY COUNT

**Consent form – PRiDE-GLP1 Sub-study**

**Study title: Role of GLP-1 Levels in gestational diabetes mellitus (PRiDE-GLP1 Sub-study)**

Participant Study ID Number: _____

Name of Midwife/Researcher taking consent: _____

Please initial box

1. I confirm that I have read the Participant Information Sheet (Version 1.2, dated 26th February 2014) for the above study. I have had the opportunity to ask questions and have them answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw from the study at any time without my medical care being affected ☐
3. I agree to give my blood samples for the study as outlined in the Participant Information Sheet. I understand that various tests will be carried out on these samples but my identity will be kept strictly confidential and the results will not be made available to me personally. I agree for these samples to be stored for future additional analyses under the guardianship of University of Warwick after the end of the study ☐
4. I give permission for relevant sections of my medical notes and data collected during the study to be looked at by authorised researchers, individuals from regulatory authorities or the NHS Trust, where it is relevant to the study ☐
5. I agree for my General Practitioner and Obstetric team (including doctors and midwives) to be informed of my participation in this study ☐
6. I agree to take part in the study ☐

| Name of Participant (Print) | Signature of Participant | Date |
|---|---|---|
| _____ | _____ | _____ |
| Name of Midwife/Researcher | Signature of Midwife/Researcher | Date |
| _____ | _____ | _____ |

George Eliot Hospital NHS NHS Trust

Consent form (PRiDE-GLP1) ver. 1.2_ 26th Fenruary 2014

## A3.3 Diprotin A preparation and blood sampling protocol

### PRiDE-GLP1 sub-study sample collection protocol

**Calculations**

- Need Diprotin A 35ul/ml of plasma (round up to 40ul/tube) – final concentration 0.1mM/ml plasma
- Solubility: 1mg/ml
- For 10 participants, need (5x10) = 50 tubes with total of (50 x 40ul) = 2000ul or 2ml

**To make-up**

- 2ml Mili-Q water
- Weigh out 2mg Diprotin A powder
- Add powder to small Falcon, then water to make up 2 ml
- Dissolve well
- Aliquot into 10 Eppendorfs of 200uL each (or equivalent for total amount)
- Store at -20$^{o}$C until use

**Protocol**

**Before blood collection**

- Require plasma sample x1 (EDTA tube, purple-top) and serum sample x1 (Plain tube, yellow-top) at each time point during GTT (i.e. 0, 30, 60, 90 and 120 mins)
- Remove DPP-4 inhibitor (Diprotin A) from freezer to thaw on the bench. Vortex for 10 seconds before use.
- Add 40ul Diprotin A to EDTA tube before blood is taken and store tube at 4$^{o}$C until use

**After blood collection**

- Draw additional 5 mls of blood for GLP-1 sub-study every 30 mins during GTT (i.e. 0, 30, 60, 90 and 120 mins)
- Divide into 1 EDTA and 1 Plain tube which have been prepared earlier (2.5mls in each)
- Invert tubes 8-10 times to allow thorough mixing but do not vortex
- Spin tubes at 20$^{o}$C, 2000 rpm for 15mins
- Aliquot plasma and serum into 2 vials each (at least 500ul in each vial)
- Label with Study ID, time point (GLP 0/30/60/90/120 corresponding with time of sampling during GTT) and date

# A3.4 PRiDE-GLP1 sub-study ethics approval

Revised 11/12/2014 to correct document version numbers and dates

NHS
Health Research Authority

National Research Ethics Service

**NRES Committee West Midlands - South Birmingham**
HRA NRES Centre Manchester
3rd Floor
Barlow House
4 Minshull Street
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M1 3DZ

Tel: 0161 625 7815
Fax: 0161 625 7299

28 February 2014

Dr Ponnusamy Saravanan
Associate Clinical Professor & Honorary Consultant Physician, Diabetes, Endocrine & Metabolism
University of Warwick & George Eliot Hospital
Clinical Sciences Research Laboratories
Warwick Medical School (UHCW Campus)
Coventry
CV2 2DX

Dear Dr Saravanan

| | |
|---|---|
| **Study title:** | **Micronutrients in Pregnancy as a Risk Factor for Diabetes and Effects on mother and baby: an MRC-funded study (PRiDE)** |
| **REC reference:** | **12/WM/0010** |
| **Amendment number:** | **Amendment 3** |
| **Amendment date:** | **31 January 2014** |
| **IRAS project ID:** | **90943** |

- The amendment consists of a sub-study to be carried out on a selection of participants from the main study.

The above amendment was reviewed by the Sub-Committee in correspondence.

**Ethical opinion**

- The sub-committee requested that the following bedded to the Participant Information Sheets 'If you would like to speak to someone independent about taking part in this study please contact the Patient Advice and Liaison Service at [INSERT LOCAL PLAS CONTACT DETAILS]'

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

| Document | Version | Date |
|---|---|---|
| Notice of Substantial Amendment (non-CTIMPs) | Amendment 3 | 31 January 2014 |
| Protocol | GLP-1 Sub-Study - Version 1.2 | 16 January 2014 |
| Participant Information Sheet: Main Study | 3.1 | 26 February 2014 |
| Covering Letter | | 31 January 2014 |
| Participant Consent Form: GLP-1 Sub-study | 1.2 | 26 February 2014 |
| Summary/Synopsis | 4.0 | 16 January 2014 |
| Participant Consent Form: Main Study | 3.1 | 26 February 2014 |
| Participant Information Sheet: GLP-1 Sub-study | 1.2 | 26 February 2014 |

**Membership of the Committee**

The members of the Committee who took part in the review are listed on the attached sheet.

**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

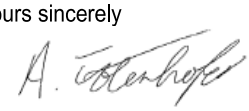
**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

| **12/WM/0010:** | **Please quote this number on all correspondence** |
|---|---|

Yours sincerely



Signed on behalf of:
**Professor Simon Bowman**
**Chair**

E-mail: nrescommittee.westmidlands-southbirmingham@nhs.net

*Enclosures:* *List of names and professions of members who took part in the review*

*Copy to:* Ms Donna McLean - Chelsea and Westminster Hospital NHS Foundation Trust

Dr Peter Hedges - University of Warwick

## Appendix 4 – Materials for laboratory experiment (Chapter 9)

### A4.1 Solutions for Western blot

#### 4.1.1 Running buffer

900ml distilled water ($dH_2O$)
100ml tris-glycine 10x
1g sodium dodecyl sulphate (SDS) (final concentration 0.1%)

#### 4.1.2 Transfer buffer

700ml $dH_2O$
200ml methanol
100ml tris-glycine 10x

#### 4.1.3 Phosphate buffer saline – Tween (PBS-T)

900ml $dH_2O$
100ml PBS 10x
1ml Tween

#### 4.1.4 I-block

200m PBS-T
400mg I-block (final concentration 0.2%)
Stirred on gentle heat until well dissolved

#### 4.1.5 10% Resolving gel (per 2 plates)

7.8ml $dH_2O$
6.6ml acrylamide
5.2ml resolving buffer
200ul APS
20ul TEMED

#### 4.1.6 4% Stacking gel (per 2 plates)

6.1ml $dH_2O$
1.3ml acrylamide
2.5ml stacking buffer
50ul APS
10ul TEMED

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